

**Control of
Cell Division and the
Induction of Cancer**

MONOGRAPH 14



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INTERNATIONAL SYMPOSIUM
ON THE CONTROL OF CELL DIVISION
AND THE INDUCTION OF CANCER

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and
Cali, Colombia
July 1-6, 1963

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and Pablo Mori-Chavez, M.D.

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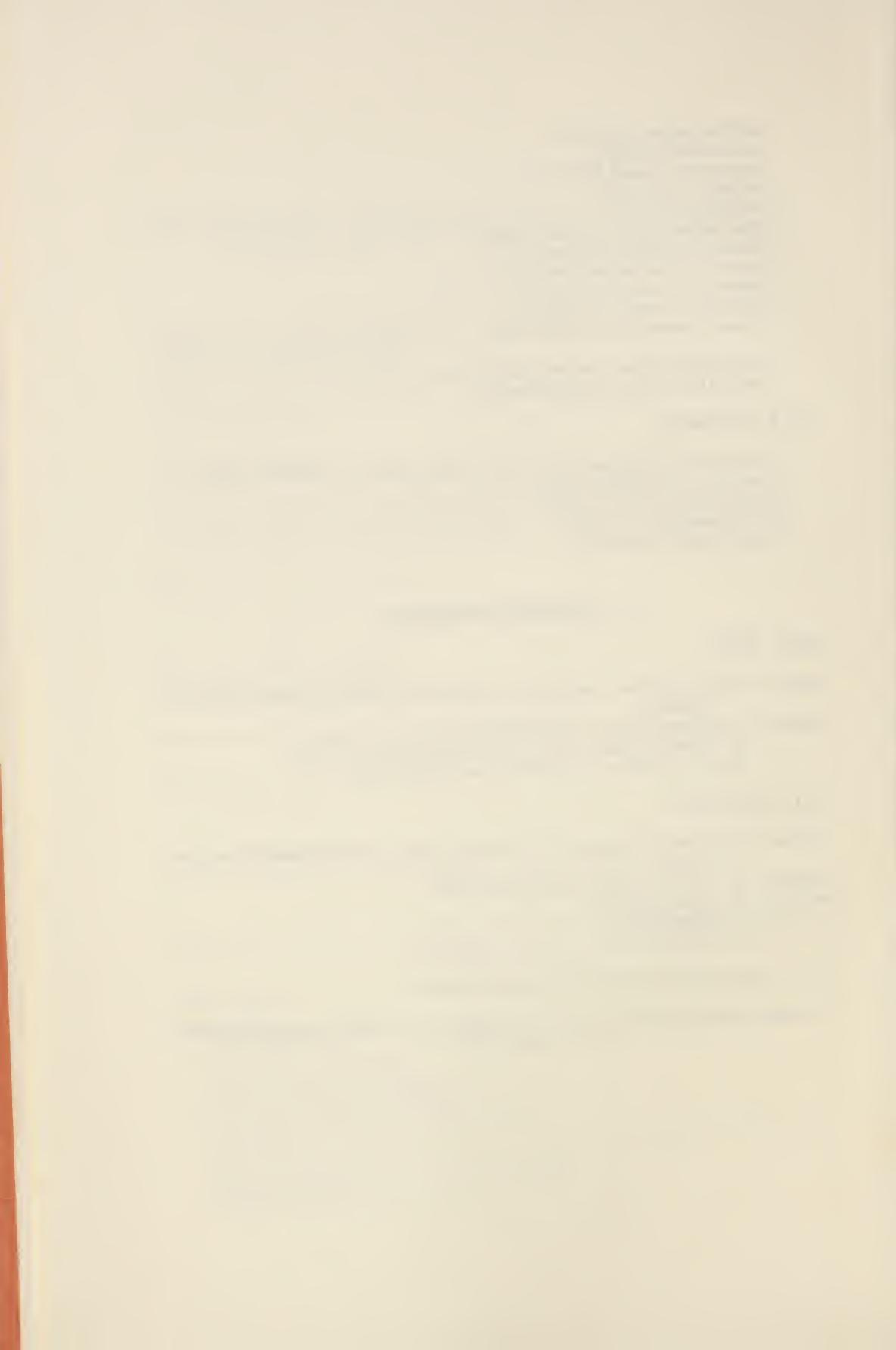


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Introduction¹

ALEXANDER HOLLAENDER, *Biology Division, Oak Ridge National Laboratory,² Oak Ridge, Tennessee*

THE INTERNATIONAL Symposium on the Control of Cell Division and the Induction of Cancer, held at Lima, Peru, and Cali, Colombia, July 1 to 6, 1963, was the third in a series of symposia organized in cooperation with our Latin American colleagues to emphasize basic aspects of biology.

The first conference was held in 1961 at Santiago, Chile, on "Tissue Transplantation" and the second in 1962 at São Paulo and Rio de Janeiro, Brazil, on "Mammalian Tissue Culture and Cytology" and "Specific Topics in Radiobiology."

All scientists attending these symposia have profited, those from Latin America as well as from other parts of the world. Personal relationships, so useful in international cooperation in the sciences, have been established at these meetings.

The possibility of holding the present symposium was first discussed with Dr. Pablo Mori-Chavez and others in Lima. It seemed that the basic aspect of cancer induction and malignant growth was an appropriate topic because of recent interest and advances in this field. It also seemed appropriate to discuss some of these fundamental questions at Lima, a place we consider the cradle of American learning. Less than 60 years after the discovery of America a strong center of learning was established there which holds for the whole world a certain intellectual glamor that can serve as a focal point of interest on the development of Latin American science.

The second meeting place, The University of Cali Medical School, is one of the newest medical schools in Latin America. It has already established itself as a most important one. An encouraging sign in Lima and Cali, as well as in other places in Latin America and throughout the world, is that fact that the young scientists—and by "young" I do not mean chronological age but young in spirit—are not always willing to put up with forces which retard scientific efforts, but seek newer lines of scientific research and methods for obtaining them.

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

The meeting in Lima was opened by Dr. Oscar Soto, the President of the symposium, who is Vice-Rector, Universidad Peruana de Ciencias Médicas y Biológicas. I made a few introductory remarks and also delivered the greetings of the President of the National Academy of Sciences, Dr. Frederick Seitz, who expressed the deep interest of the National Academy in these cooperative symposia. Dr. Daniel Mazia, of the University of California, gave a short address in memory of the late Professor Eleazar Guzman Barron from Peru who was associated with the University of Chicago and was a very active investigator on the biological effects of radiation. An inaugural address in the opening ceremonies was given by General Victor Solano Castro, the Peruvian Minister of Public Health.

The meeting in Cali was opened by Dr. Gabriel Velasquez Palau, Decano, Facultad de Medicina, Universidad del Valle. Dr. Mario Carvajal, Rector, Universidad del Valle, gave a welcome address, and a few words of welcome also were presented in the name of the foreign guests. In both places the papers were given in English; however, translators were available. Most of the discussions were recorded, and a summary of these discussions follows each paper in this volume.

The success of the Lima-Cali conference was made possible through the untiring efforts of Dr. Pablo Mori-Chavez, the general secretary of the symposium, and Dr. Pelayo Correa, secretary of the organizing committee in Cali. Dr. C. C Congdon acted as consultant to the organizing committees. Besides the group of investigators from Latin America and the United States, scientists from Great Britain and Canada made important contributions.

The symposium would not have been possible without the financial support from governmental, commercial, and private groups in Peru and Colombia and the generous contribution of many United States and international governmental agencies and philanthropic organizations.

This introduction and several other parts of the volume are printed in Spanish in addition to English; these portions include abstracts of each paper and of the summary by Dr. Congdon.

PROLOGO

El Simposio Internacional sobre el Control de la División Celular y la Inducción de Cancer que tuvo lugar en Lima, Perú, y en Cali, Colombia, del 1º al 6 de Julio de 1963, fué el tercero de una serie de simposios organizados en cooperación con nuestros colegas latino-americanos para acentuar aspectos básicos de biología.

La primera conferencia tuvo lugar el año 1961 en Santiago de Chile sobre "Transplantes de Tejidos," y la segunda el año 1962 en San Pablo y en Rio de Janeiro sobre "Cultivo de Tejidos y Citología de Mamíferos" y "Temas Específicos de Radiobiología."

Todos los científicos que han asistido a estos simposios, tanto los de Latino América como los de otras partes del mundo, han obtenido

provecho. Relaciones personales, tan útiles en la cooperación internacional en las ciencias, se han establecido en estas reuniones.

La posibilidad de realizar el presente simposio fué primero discutida con el Dr. Pablo Mori-Chávez y otros en Lima. Se pensó que los aspectos básicos de inducción de cancer y del crecimiento maligno sería un tema adecuado debido al interés reciente y a los avances en este campo. También se consideró la conveniencia de discutir algunos de estos aspectos fundamentales en Lima, un lugar que consideramos la cuna de la enseñanza americana. Menos de 60 años después del descubrimiento de América un eficiente centro de enseñanza fué establecido allí que mantiene en todo el mundo cierto brillo intelectual que puede servir como un punto focal de interés para el desarrollo de la ciencia en Latino América.

El segundo lugar de la reunión, la Facultad de Medicina de la Universidad de Cali, es una de las más recientes escuelas de Latino América, y ya se ha constituido en una de las más importantes.

Un signo alentador en Lima y Cali, así como en otros sitios de Latino América y de todo el mundo, es el hecho de que los jóvenes científicos—y al decir “jóvenes” no me refiero á la edad cronológica sino a los jóvenes de espíritu—ya no se conforman con las fuerzas retardatarias del progreso científico, y procuran nuevas líneas de investigación científica y los métodos para alcanzarlas.

La reunión de Lima fué abierta por el Dr. Oscar Soto, el Presidente del Simposio, quien es Vice-Rector de la Universidad Peruana de Ciencias Médicas y Biológicas. Yo dirijí unas palabras de introducción observando ciertos enfoques de este simposio y también dí lectura al saludo enviado por el Presidente de la Academia Nacional de Ciencias de los Estados Unidos, Dr. Frederick Seitz, quien expresó el profundo interés de la Academia Nacional en estos cooperativos simposios. El Dr. Daniel Mazia de la Universidad de California consagró algunas frases a la memoria del Dr. Eleazar Guzmán Barron del Perú quien estuvo asociado con la Universidad de Chicago y fué un destacado investigador sobre los efectos biológicos de la radiación. El discurso de inauguración fué dado por el General Victor Solano Castro, Ministro Peruano de Salubridad Pública.

La reunión en Cali fué abierta por el Dr. Gabriel Velasquez Palau, Decano de la Facultad de Medicina, Universidad del Valle. El Dr. Mario Carvajal, Rector de la misma universidad dió el discurso de bienvenida, así mismo como palabras de bienvenida para los huéspedes extranjeros. En los dos lugares los trabajos fueron presentados en Inglés, sin embargo, tenían traducción simultánea á disposición. La mayoría de los escritos fueron anotados y un sumario de estas discusiones prosigue cada artículo en este tomo.

El éxito de las conferencias en Lima y Cali fué posible por los esfuerzos del Dr. Pablo Mori-Chávez, secretario general del simposio, y del Dr. Pelayo Correa, secretario del comité organizador en Cali. El Dr. C. C. Congdon actuó como consultante del comité organizador. Además del grupo de investigadores de Latino América y de los Estados Unidos,

hombres de ciencia de la Gran Bretaña y del Canadá hicieron importantes contribuciones.

El simposio no se hubiera podido realizar sin el apoyo financiero gubernamental, kommercial y de varios grupos particulares del Perú y Colombia, y sin las generosas contribuciones de varias agencias y organizaciones gubernamentales y filantrópicas Norte Americanas é internacionales.

Esta introducción y varias otras partes de éste volúmen han sido publicadas tanto en Castellano como en Inglés; éstas partes incluyen abstractos de cada labor y del sumario del Dr. Congdon.

Control of Cell Division

Cell Division and a Hypothesis of Cancer^{1, 2}

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SUMMARY

The hypothesis is made that cancer can result from a loss of intercell control of cell division. Some known mechanisms of cell regulation are cited. From these studies, it is clear that special regulatory mechanisms frequently arise in living cells. Metabolic processes can be controlled by small molecules which are not chemically similar to the molecules involved in the metabolic process itself. Some aspects of the biochemical regulation of cell division are briefly discussed. It is concluded that some cytoplasmic event must occur to initiate cell division. In higher multicellular organisms, the essential event seems to depend on extracellular factors. The

evidence for hormone-like compounds in the circulation which regulate specific cell divisions is referred to. The continuing cell growth so often observed in cancerous cells is postulated to depend on a loss of regulation by substances external to the cell. It is suggested that these inhibitors of cell division are prevented from entering cancer cells, owing to altered surface properties. The altered surfaces of the cancer cells are supposed to no longer permit entry of the growth inhibitory substances. Some information relating to the modified surfaces of cancer cells is cited.—Nat Cancer Inst Monogr 14: 7-20, 1964.

JUST AS the growth of the whole organism depends on a carefully controlled balance of its parts, so the growth of a living cell depends on a controlled balance of the biochemical reactions involved in its synthesis. Scientists have recently begun to understand some of the mechanisms that govern the regulated syntheses of essential metabolites in living cells (1). These mechanisms include inductions and repressions, which determine the amounts of enzymes required to catalyze critical reactions in the cell. Also there are the feedback mechanisms of enzyme inhibition and enzyme activation, which determine how rapidly the enzymes can operate. Other mechanisms involve the more gradual reversible or irreversible activation and inactivation of key enzymes, making them available when needed or removing them when

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

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they are in excess. Furthermore, specific mechanisms for transporting compounds into cells have been discovered. These, permeases, probably are important in the transport not only of nutrients into the cell, but also of hormones and growth-regulating substances.

From investigations on metabolic control, it is generally concluded that mechanisms have developed which are specifically designed for control in living cells. These mechanisms are not involved in the actual catalytic activity, but are purely regulatory processes limiting the rates of the reactions. Undoubtedly, they developed in response to the organism's requirements for survival. They have their parallels in the whole organism in such oddities as horns, poison glands, and protective colors, all designed to help in the survival and greater fitness of the whole organism.

Since special controlling mechanisms evolve according to needs at the subcellular and whole organism levels, the logical question is whether similar controls can be discovered for the fundamental processes occurring at the cell level. These processes are cell division and the cessation of cell division when growth is complete, which involves us in the problem of *unregulated* growth of cells that is basic to cancer (2). Perhaps the most fundamental characteristic of cancer is that the cell fails to stop dividing, while a normal cell's growth is limited. It has been suggested often that this defect in the cancer cell is the result of the breakdown of some fundamental mechanism that normally controls and limits cell division (3-5).

In this paper we will: 1) briefly outline current information on mechanisms that control growth; 2) consider the biochemistry of cell division and its limitation; and 3) speculate briefly on how these cell division mechanisms can break down in cancer cells.

The hypothesis is suggested that specific compounds acting between cells determine whether or not cells divide. The failure of cancer cells to permit penetration of these compounds might be responsible for their continued growth.

In the broad area covered in this paper we will summarize some current thoughts. In general, we will not present original references but will refer to review articles. Some parts will be frankly speculative and will be labeled as such. We feel that speculation, if properly indicated, will not be objectionable here.

METABOLIC CONTROL MECHANISMS

Enzyme Induction and Repression

Living cells synthesize certain enzymes at rates that differ by factors of ten or even as much as a thousand, depending on whether or not specific compounds are provided as nutrients. If the added specific compound stimulates enzyme formation it is called an inducer; if it inhibits

enzyme formation it is called a repressor. Enzyme induction and repression can be important in regulating the amount of an enzyme available for a specific catalysis. The best known example, addition of the sugar lactose to a growing population of the bacterium *Escherichia coli* causes an immediate 1000-fold increase in the rate of β -galactosidase formation. The increase in this enzyme in turn greatly stimulates the rate at which lactose is used as a carbon source by the bacteria. As an example of enzyme repression: The stimulation of synthesis of several enzymes in the pathway of arginine biosynthesis, when arginine is no longer supplied as a nutrient to the bacteria, results in an increased rate of synthesis of arginine.

These nutritional inducers and repressors are believed to act because they either inhibit or activate an internal large-molecular-weight molecule, also named a repressor, that blocks synthesis of the corresponding enzyme (6). Thus, addition of an inducer makes the repressor less active; consequently, enzyme formation is less inhibited. An inducer is an inhibitor of an inhibitor. We will not go into the genetic-type experiments that provide evidence for these ideas, but will merely try to summarize some thoughts derived from them which are perhaps useful in considering cell division.

The first conclusion is that cells contain at least two types of genes. One type, the structural genes, contains information specifying the structures of enzyme molecules. The other type does not specify the structure of the enzyme, but the degree of activity of the structural gene. When these regulatory genes are eliminated by mutation the cell may still grow, but not as efficiently as it did originally.

The second conclusion is that the small molecule—the nutritional inducer or repressor—does not have to react with the enzyme whose formation it affects; *i.e.*, the specificity of control does not have to resemble the specificity of enzyme action. Therefore, compounds quite unlike the ones that are being modified in metabolism by the enzyme can greatly affect the synthesis of the enzyme. This suggests that regulation of one pathway can be controlled by the products of other pathways, or indeed by substances such as hormones from other parts of multicellular organisms.

Feedback Inhibition and Activation

Small molecules not only affect the rate of enzyme formation, but also can either inhibit or stimulate the *activity* of enzymes. The feedback inhibition in the pyrimidine pathway is one of the best examples of this type of control (7). Here, an end product of the pathway, cytidine triphosphate, is a potent inhibitor of the first specific reaction of the entire pathway. This step, the conversion of aspartic acid to carbamyl aspartate, is many metabolic steps removed from the reaction which produces cytidine triphosphate. As a result of this inhibition, excess pyrimidine nucleotides within the cell prevent formation of more of these compounds; therefore the cell does not overproduce them.

The feedback inhibitor, cytidine triphosphate, inhibits the enzyme when it combines with a special regulatory site on the enzyme molecule. This site is quite distinct from the sites at which the substrates of the enzyme are bound. It has been named an allosteric site (8). In this mechanism a parallel to the mechanism of regulation of enzyme formation is seen. The cell has created two kinds of loci: One is a functional locus for enzyme activity, and the other is a regulatory locus for control of this activity. One can separate these two loci and obtain enzymes with uncontrolled activities. We see again, at this level of organization, that nature devises specific mechanisms for control.

The regulatory small molecule—the feedback inhibitor—is not structurally related to the substrate on which the enzyme acts. This again stresses the possibility of control of a reaction by compounds in different metabolic pathways or from different parts of a higher organism.

Regulation by Enzyme Activation and Inactivation

These regulatory mechanisms of enzyme induction-repression and feedback inhibition have often been reported in bacteria, and less frequently in higher organisms. Another sort of mechanism is quite frequently found in the multicellular organisms (9). This is a control of enzyme amount by activation and inactivation of pre-existing molecules. One example of this type is the activation of the enzyme phosphorylase. It exists in two forms, one of which is far more active than the other. One form can be converted to the other by specific activating and inactivating enzymes. These changes involve actual chemical reactions of addition or removal of a phosphate group on the enzyme.

An example of a less drastic change is the activation of the first enzyme in fat synthesis of animals. This enzyme exists in two forms, one of which is a monomer and the other a trimer. Citric acid converts the monomer into the trimer during a period of about one-half hour. Thus the level of active enzyme, the trimer, and rate of formation of fat depend on how actively the organism is producing citric acid or some related compound.

We must also mention the activation of mammalian glutamic dehydrogenase by a variety of small molecules, including some hormones. Again the regulatory small molecule does not have any chemical structural resemblance to the substrates of the enzyme. It probably acts on a special site designed for control of this enzyme. The hormones change the molecular weight, and in doing so change the enzyme's activity. These experiments suggest that sometimes hormones may act by activation of the enzyme molecules, through combination with specific regulatory sites.

Permeability Mechanisms Involved in Control

For many years it has been known that some nutrients are carried into living cells by active transport mechanisms that can increase the concen-

tration of the material inside the cell far above its outside concentration. Furthermore, some of these transport mechanisms, called permeases, are inducible (10); that is, their amounts increase greatly under suitable nutritional conditions. Permeases might carry not only foods into the cell but also regulatory molecules. Conversely, it is thought that some hormones regulate metabolism by modifying the permeability of cells to nutrients (11, 12).

As a consequence of the action of all these controlling mechanisms, we can imagine a harmonious functioning of the metabolism of living cells. Permeases and inducible mechanisms can permit foodstuffs to be utilized when they are available. Repression and feedback mechanisms control the synthesis of enzymes and the formation of end products according to need.

CELL GROWTH AND CELL DIVISION

Growing cells increase in mass, divide their nuclear material, and divide into daughter cells. Each of these events seems to depend on the preceding one. In spite of numerous experiments with many organisms, well reviewed by Swann (13, 14) and by Lark (15), one can only conclude generally that some event must occur in the cytoplasm before the deoxyribonucleic acid (DNA) synthesis necessary for nuclear division can commence. That cell division should follow nuclear division seems logical, no matter what the mechanism. Normal cell division requires that each offspring must receive a complete complement of genetic material. This is possible only after the genetic material has duplicated and the nuclei have separated.

Some information can be obtained from bacteria, which serve as models for many processes of cell regulation. Bacteria undergo a regular cycle of growth and division as long as nutrients are available and toxic products are absent. Cell division appears to be a final event of the division cycle, and depends on the prior increase of cell mass and division of the nuclei. Once the nuclei have divided, the laying down of a septum, from the cell surface inward, occurs between them. This septum deposits a cell wall separating the original cell into two daughters (16).

The more basic question is how the doubling of the nuclear material and the cytoplasmic parts of the cell are coordinated within the time corresponding to one cell division. No mechanism is known for this coordination. Rather, experiments with inhibitors or rapidly altered growth conditions show that DNA synthesis is easily uncoupled from the other syntheses in the cell.

DNA is made during only a portion of the cell division cycle in animal and plant cells. Similar observations have been made with bacteria, though DNA synthesis occupies most of the cycle under usual growth conditions. These results show that the synthesis of DNA and the other parts of the cell are not closely coupled because cytoplasmic synthesis goes on continually, but DNA synthesis does not.

Synthesis of the bacterial chromosome begins in a highly specific way. It is duplicated as a unit, as shown by several recent experiments [see (16)]. Duplication starts at one end of the bacterial chromosome and continues along the chromosome until it is completed. Replication of DNA only once during a cycle of cell division might therefore depend on the creation of a unique set of conditions within the cell at only one time during the cycle.

What then is this event in the cell that initiates DNA synthesis? Lark (15) has discussed this problem at length. Only fragments of information are available at present. Synchronous division of nuclei in a common cytoplasm of slime mold suggests that a special condition in the cytoplasm is necessary. Also, the fact that the size of a cell depends on the ploidy, or number of copies of nuclear material it contains, suggests that nuclear duplication depends on some ratio of nuclear and cytoplasmic factors. The surface-to-volume ratio might be important. But the formation of long filamentous forms of bacteria suggests that separation of the cells is not vital for nuclear duplication.

Some experiments with bacteria suggest that this special event for initiating DNA synthesis requires protein synthesis. Aside from this, the author is not aware of any biochemical evidence regarding the nature of this controlling condition.

Admittedly, knowledge of the biochemical basis of cell division is in a very primitive state. We can only imagine that as the newly divided cell grows its internal conditions periodically change, and at some point the formation of some mechanism for commencing the duplication of the genetic material is set into motion by alterations within the cytoplasm. The alteration might be highly specific for cell duplication. This is by analogy with the high specificity of mechanisms for much less vital regulations, of processes such as enzyme formation. How this regulatory machinery is activated remains a mystery.

TERMINATION OF CELL DIVISION

Bacteria and other single-celled organisms seem to continue to divide until conditions become unfavorable, perhaps due to lack of nutrients or oxygen. Plant cells cease division because of formation of a rigid cell wall that stops growth, although this may not be the principal or fundamental reason for the division cessation.

In higher animals, cells of some tissues continue dividing throughout the life of the animal. Other cells divide rapidly until the organ in which they are located reaches a definite size, and then divide much more slowly. Other cells cease division when the animal has reached maturity. Clearly, the regulatory mechanisms operate differently in various cell types.

The mechanism which stops cell division is not one that simply stops the synthesis of large molecules in general. Various nondividing animal

cells, such as liver and pancreas, can produce proteins in large amounts. Neither does the mechanism simply stop DNA synthesis; cells in which this occurs selectively grow to giant size (15).

The ability of mature animal cells to divide is not lost permanently. Striking examples of recovery of the ability to divide are seen when some cells of a tissue are removed. For example, if a large part of the liver is taken away, the remaining cells soon start to divide rapidly. Wounds bring on a rapid cell division to restore the missing tissue. These proliferations seem to be highly specific, and suggest that the loss of cell division depends on the relation *between* cells and not on some property found within the cells (17, 18).

The stimulation of cell division, when some cells are removed, may not only affect the cells immediately next to them but also others, *e.g.*, removal of one kidney of an animal causes an increase of cell division in the other kidney. Removal of the liver of one of a pair of animals with a common circulation (parabiotic animals) causes stimulation of cell division in the other animal. We must imagine a hormone-like effect that is specific for each cell type and which is carried throughout the entire animal.

Materials in the blood which influence cell growth and cell division have been observed quite often. These include compounds inhibiting cell growth and compounds stimulating it; sometimes both effects are found in the same tissue. Different hormones also are known to have both kinds of effects (19).

A most elegant hypothesis of the regulation of growth has been suggested by Weiss (20). Each cell type is postulated to contain a specific growth stimulatory substance, which remains inside the cell that forms it. The cell also produces a growth inhibitory compound which can escape from the cell. Whether growth occurs or not depends on the relative amounts of the intracellular growth stimulatory material and the concentration of the inhibitor, resulting from production within the cell and also from the balance of escape and entry of the material between cell and circulation.

It now is possible to postulate that both specific growth inhibitory and growth stimulatory substances are produced by cells [*e.g.*, (21)]. By analogy note a competition of inhibitor and stimulator in the aggregation of slime molds (22), the development of insects (23), and the development of plant tissue cells, especially plant tumors (24).

CANCER CELLS VERSUS NORMAL CELLS

Surveys of the biochemistry (25) and biology (2-4) of cancer suggest that many differences exist between cancer cells and normal cells. These differences are found in the metabolism, internal cell structure, antigens, on the cell surface, etc. These changes are not all fundamental; many of them occur as secondary consequences during the evolution of the cancer cell. Only a few differences are found in all tumors; presumably these are the most basic to the development of malignancy. Biochemical hypoth-

eses have generally assumed that a defect in one key enzyme is responsible for cancer.

This idea is perhaps best illustrated by considering the evolution of hypotheses regarding metabolic differences between cancer cells and normal cells (26). For many years, starting with Warburg's hypothesis of a change in glucose utilization by cancer cells (27), differences in the enzyme content of normal and cancer cells were sought. Many differences were found. More recently, however, it has become evident that these differences do not exist in all cancers but rather that the more frequently a cancer is subcultured the more it deviates from the normal cell. This has led to Potter's minimal deviation postulate, which states that only those changes found in all cancers are really fundamental to the development of malignancy (5). Cancers have been found whose enzyme content do not differ in any known way from the normal cells from which they originated. It is now suggested that the fundamental difference is not one of loss of an enzyme, but rather loss of a mechanism for controlling the amount or possibly the activity of a crucial enzyme involved in cell division (26).

Cancer as the Loss of a Regulatory Mechanism

For many years it has been suggested that cancer results from the loss of a mechanism controlling cell division. The hypotheses as to what this mechanism might be have been numerous, but no evidence gives clear support to any one idea. Broadly speaking, we can imagine that the lost control depends on: 1) a loss of specificity at some site within the cell influenced by a controlling metabolite, 2) a failure of the controlling metabolite to be produced, or 3) a failure of the controlling metabolite to reach the controlling site.

If we accept the notion that cancer is not basically a defect *within* a cell, but rather represents a decreased *intercellular* response to growth control by neighboring cells, some choice can be made between these possibilities. This is a reasonable surmise; several investigators have noted that cancer cells lose their responsiveness to neighboring cells (28, 29). Eventually they become completely independent; they grow and divide under a variety of conditions—even in hosts—in which normal cells do not grow. These results suggest that cancer cells no longer respond to the growth inhibitory substances produced by their neighbors (14).

Loss of intercellular control would seem to rule out the second possibility, *i.e.*, failure of the controlling metabolite to be produced.

Most biochemical models assume a loss within the cell, as mentioned earlier. But a frequent suggestion is that changes of the cell surface involve loss of antigenic sites that are essential for the control of cell growth (3, 4, 30).

Kalckar discusses (*see* Kalckar, this Symposium) differences in cancer and normal cell surfaces that correlate with low levels of an enzyme

(epimerase) and high rates of glycolysis. Perhaps these hypotheses of loss of an intracellular function and loss of entry are not altogether different.

For speculation on the primary defect in cancer cells, it is worth noting that development of cancer seems to be a gradual event (28). The induction of cancer by ultraviolet light or carcinogens is a cumulative consequence of many doses (31). A carcinogen first reversibly causes cells to lose their growth inhibition controls, before they actually become cancerous (32).

The third hypothesis, which does not seem to have been suggested but seems to possess merit in view of current observations, is that the growth-regulating substances are unable to enter the cancer cell. The ability of these substances to penetrate the cancer cell might depend on specific permeability mechanisms that gradually might be lost from the normal cell as it is converted to a cancer cell. Such a cell would then continue to grow irrespective of the inhibitors produced by other cells surrounding it.

This hypothesis is consistent with the changed response of cancer cells to growth stimulatory and inhibitory compounds, including hormones (25, 33, 34). It is also consistent with the several observations of changed surface properties of cancer cells (2).

The Cancer Cell Surface

Berrill (35) noted that carcinogens and also parthenogenic agents, which stimulate cell division, are all surface active compounds. He suggested that an effect on the cell surface might be involved in the induction of cancer.

Hormones are well known to affect the cell surface. They can modify permeability of the cell to various metabolites (12). Hormones can also be carcinogenic (36). After the cancer is established it is in some cases dependent on hormones, or its growth can be to some extent controlled by hormones (37). The evidence suggests a close relation between cancer and the action of these substances at the cell surface.

A variety of direct observations show differences between the surfaces of normal and cancer cells (38). The surface charges of normal and cancer cells differ, as shown by data on their mobility in an electrical field. Their charge changes progressively as the cancer cells are subcultured (39). This result suggests that some of the surface differences might be secondary events in the development of a cancer cell.

Direct observations show structural surface differences of normal and cancer cells. Cancer cells do not adhere to one another as strongly as do normal cells. However, they tend to adhere loosely, more easily to other cells. Normal cells inhibit the movement of their neighbors, by what is called contact inhibition, more strongly than do cancer cells. In general, the physical interactions of cancer cells are diminished.

Much information has been accumulated regarding the antigens on the surfaces of normal and cancer cells (30, 40). Several hypotheses of cancer have developed from these observed differences. The antigenic differences are striking and are undoubtedly important in the functioning of cancer cells.

Permeability Changes of Cancer Cells

Le Breton and Moulé (25) have suggested permeability differences between cancer and normal cells on the basis of the modified action of thyroxin on the two types of cells. The release of intracellular material from tumor and normal cells is different. This suggested to Holmberg (41) that the surfaces of the two cells are different. Possibly, too, the loss of regulation of many enzymes' synthesis observed by Pitot (26) is caused by a loss of the ability of the inducing and repressing compounds to enter the cancer cell, as compared to the normal cell.

These results suggest that the cancer and normal cells indeed might differ in permeability. The observations are still not numerous; furthermore, it remains to be shown that they are fundamental to malignant growth.

RESUMEN

Se formula la hipótesis de que el cancer puede resultar de una pérdida del control intercelular de la división celular. Algunos mecanismos conocidos de la regulación celular son revisados. De estos estudios se evidencia que mecanismos regulatorios especiales surgen frecuentemente en las células vivientes. Los procesos metabólicos pueden ser controlados por pequeñas moléculas que no son químicamente semejantes a las moléculas envueltas en el propio proceso metabólico.

Luego se discuten brevemente aspectos de la regulación bioquímica de la división celular. Se concluye que algún cambio citoplásmico debe ocurrir a fin de iniciar la división celular. En los organismos multicelulares más elevados tal evento parece depender de factores extracelulares. Se hace referencia a la evidencia de compuestos de tipo hormonal en la circulación que regulan las divisiones celulares específicas.

El crecimiento celular continuo que se observa a menudo en las células cancerosas se asume que depende de una pérdida de la regulación debida a substancias extracelulares. Se sugiere que estos inhibidores de la división celular están impedidas de entrar en las células cancerosas debido a alteración de las propiedades de superficie. Se supone que tales superficies alteradas de las células cancerosas no permiten más la entrada de substancias inhibitorias del crecimiento. Se cita alguna información relativa a las superficies modificadas de las células cancerosas.

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DISCUSSION

Tono:* I would like to mention some work done by Dr. Seymour S. Cohen and myself (*J Biol Chem* 237: 1271, 1962) on the existence of a nucleoside permease in bacterial cells. We found that nongrowing cells of a pyrimidine-requiring mutant of *Escherichia coli* were able to accumulate 1- β -D-arabinofuranosyluracil. The cells concentrated the compound by a factor of 50, from about 0.1 μ mole per ml in the medium to about 5 μ mole per ml within the cells. We also found that cells of *E. coli* which did not require pyrimidines to grow accumulated the arabinosyluracil to a lesser extent. Another mutant, resistant to 5-fluorouracil, did not take the arabinonucleoside at all. This work might be related to the role that permeases could play in controlling cell metabolism.

There have also been recent reports (Paterson and Hori, *Canad J Biochem* 40: 181, 1962; Paterson, *ibid*, 1955), explaining the resistance of some Ehrlich tumor cells to 6-mercaptopurine by the failure of this compound to enter the cells. This is a cryptic phenomenon because even though the cell-free extract had all the enzymes to utilize the 6-mercaptopurine, the whole cell did not take it up.

It might also be of interest to mention that the arabinosyluracil was obtained by a biosynthetic procedure. The enzyme uridine phosphorylase splits the arabinonucleoside to give uracil and D-arabinose-1-P. The latter compound, as well as a sample of D-arabinose-1-P obtained by chemical synthesis by Dr. Khorana, can be trans-

formed into arabinosyluracil in the presence of uracil and uridine phosphorylase. Since *D*-arabinose-1-P and free bases may exist in bacterial cells together with nucleoside phosphorylases, it would then seem possible that small amounts of the arabinonucleosides could be synthesized *in vivo* by the cells. These compounds might be of importance in controlling the metabolism—within a cell or from one cell to another.

Our data point to the arabinosyluracil permease as a constitutive enzyme. However, the possibility that it might be an inducible enzyme has not been ruled out.

Pardee: Perhaps the speculations I made regarding modified permeases and cancer would sound a little more plausible if I told you several things about permeases in bacteria. Permeases can determine growth and metabolic activities. They can vary in amount, owing to a number of causes. As Dr. Tono said, they are often inducible; that is, they can be created under special nutritional circumstances as can enzymes. They do not exist under all conditions. In other words, the amount of a permease can depend on the environment, increasing or decreasing at different times.

One can observe long-lasting changes in the amount of a permease, depending on a temporary nutritional event which turns the synthesis off or on. Transient local conditions—a shock to the cell, such as exposure to some high concentration of metabolites—can change permeability from that time on. Furthermore, mutations can alter the ability of cells to produce permeases.

Kaplan: I think that we should be skeptically receptive to any new ideas that are offered to us, because our insight in the field of cancer research tends to develop tunnel vision. One area that relates to your speculations about cancer is diagnosis. In my view, the most striking advance that we have made in the diagnosis of cancer in many years is the Papanicolaou exfoliative cytology test, which depends on the fact that cancer cells have altered surface properties and decreased mutual adhesiveness. As I understood your remarks, you mentioned the possibility that surface changes might work on the basis of an essentially one-way system in which cell growth inhibitors would come from the outside, and a cell which was not permeable to these would continue to grow. However, it appears that your theory would not account for the converse situation, which is best documented for the endocrine tumors in which sustained growth stimulation, rather than inhibition, is supplied from outside the cell, leading ultimately to neoplasia.

I would like to ask you to comment further on where the regulatory mechanisms that you were talking about are located inside the cell. We are used to thinking of the cellular control mechanisms as being far inside the membrane, and it comes as a little bit of a shock to think of the fundamental change in cancer cells being located on the membrane.

Pardee: If one assumes that the growth rate of a cell depends on a balance between effects of growth stimulators and growth inhibitors, an increased permeability to the former could give a result similar to decreased permeability for the latter.

I believe the evidence from bacterial permeability studies suggests that the permeability mechanisms are in the cell membrane. I am not certain how much evidence there is regarding the location of permeability mechanisms in animal cells, but certainly the membrane or cell surface would seem to be the plausible place for their location.

Question: What controls the permeases?

Pardee: Permease amounts are variable depending on nutrition. These nutritional effects depend on genes (see Kepes and Cohen, *The Bacteria*, vol. 4, 1963).

Totter:* I would like to return to some examples of control, and describe one which I think is inherently simpler than any of those which have been previously encountered by biochemists. This example arose in the work of Dr. Washington De Angelis of the Faculty of Medicine of Montevideo while he was at the University of Georgia. He was studying a hydrogen transfer enzyme which does not react with oxygen but does with a dye, so that we have the usual series of hydrogen transfer reactions to

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the dye and from the dye to oxygen. Of course, the rate would be dependent on the oxygen concentration since the dye will cycle and oxidize the substrate. When the kinetics of this reaction were studied it was found that if one followed the oxygen concentration as it diminished, the rate of formation of end product dropped off, as you would expect, but when the oxygen concentration reached a critical low level, it sped up very rapidly, until the oxygen was all gone, then of course stopped. This, in a sense, is kind of a model for erythropoietin because it is something in which production is sped up when the oxygen tension is low. The explanation for it seems to be this: This dye can react in radical form with the reduced enzyme which then reacts in the same fashion again with the dye, and thus regenerates the dye radical. This is a chain reaction, inherently capable of almost explosive velocity, except that it is controlled by a reaction in which two dye radicals react with oxygen. So we see that as long as the oxygen concentration is high, the concentration of the dye radical is low. When the oxygen concentration drops down, the rate rises, because of course the concentration of the radical intermediate rises. I think this mechanism is available to cells in general, though I do not believe it has been studied before.

Mazia: As long as we are still speculating, I do not see what is gained by naming a fundamental modification of the cell a "permeability" change. The type of permeability you are speaking about would be very likely to involve a specific acceptor or permease, not a mere hole in the cell surface. Such molecules, as you know better than I do, are subject to the same type of genetic control as are enzymes within the cell. Until we get down to actual cases, there is no real difference between the presence or absence of an enzyme system inside the cell and the presence or absence of a permease having similar restrictions of specificity on the cell surface.

Aberrations of Metabolic Patterns of Malignant Cells and Their Relevance to Cell Biology^{1, 2}

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SUMMARY

Four types of mammalian cells with malignant potentialities show a severe interference of the capacity to convert glucose to galactose (epimerase "choke"). This is presumably due solely to the aerobic glycolysis which reaches high levels in these types of cells and raises hydrogen ion concentrations as well as the levels of reduced pyridine nucleotides, alterations that are known to interfere severely with epimerase activity. The capacity to convert glucose and its metabolites into complex galactose compounds of the surface may become insufficient in rapidly growing cells if a severe choke is imposed on the epimerase activity. A significant "thinning out" of galactosyl compounds of the surface ("ekto-

polysaccharides") may have a striking effect on the neoplastic growth versus host reaction. It is known from micro-organisms that defects of enzymes involved in the biosynthesis of specific "ektopolysaccharides" permits growth but alters the "ektobiology" (losses of surface antigens, losses of virus receptor sites). Studies of enzyme patterns in neoplastic cells remain of interest. Defects or alterations of enzyme patterns in the biosynthetic pathways leading to the formation of aberrant or defective "ektopolysaccharides" may be of special relevance to the problem of malignant neoplastic growth as seen in the light of the "graft versus host" balance.—Nat Cancer Inst Monogr 14: 21-32, 1964.

THE "PRIMITIVE" but effective glucose metabolism of tumor cells is due to the persistence of aerobic glycolysis, as discovered by Warburg nearly 40 years ago (1-3). Although exceptions have been found, aerobic glycolysis in malignant tumors is indeed a conspicuous phenomenon. The rapid degradative consumption of glucose and glutamine (4) in malignant cells might force neighboring normal cells to act as "feeders" for the malignant cell (5). If the steady-state level for glucose becomes very low, the malignant cells might be favored at the expense of the normal cells. In any case, at low glucose levels a suspension of ascites

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tumor cells will bring about a loss of glutamine from brain tissue if this tissue is coincubated with ascites tumor cells (5). Replacement of the latter by kidney cells does not deprive the brain slices of glutamine (5).

GALACTOSE METABOLISM IN MALIGNANT OR POTENTIALLY MALIGNANT CELLS

Nutritional Considerations

It has been reported that strain L cells (6-8) and lymphoma cells (9) have a block in galactose metabolism; HeLa cells (10, 11) show the same type of defective galactose metabolism.

We have found that L cells (12), HeLa cells (12), Ehrlich ascites tumor cells (13), and mammary carcinoma (12) have considerable amounts of galactokinase and epimerase but only traces of transferase (7).

The physiological importance of such a transferase defect is difficult to assess. It seems likely, however, that metastases of such tumors in tissues, like liver and kidney, which metabolize galactose with great facility, might behave in a different way, depending on whether galactose or glucose is supplied as the main carbohydrate. Galactose might discriminate against the tumor cells in the same way as in transferase-defective *Escherichia coli* mutants. Accumulation of galactose-1-phosphate tends to interfere with cell growth (14, 15).

Epimerase Activity

It has recently been observed that L cell cultures and established cultures of bovine mammary gland have lost epimerase activity (8, 9). These observations were made on intact L cells as well as broken cell preparations of L cells and glandular cultures. The epimerase defect could be ascribed to somatic mutations or to various ways of "deadaptation" and these possibilities were also discussed. We have reported briefly (12) that the lack of epimerase activity in L cells and HeLa cultures is not due to a lack of synthesis of these enzymes or to the synthesis of an abnormal epimerase, but to a severe inhibition of this enzyme brought about by an abnormal cell milieu (12, 13) stemming from aerobic glycolysis and defective respiration.

INTERFERENCE WITH EPIMERASE ACTIVITY BY DPNH

We have previously reported (16-18) that mammalian epimerase (in contrast to yeast and *E. coli* epimerase) requires diphosphopyridine nucleotide (DPN) and is strongly inhibited by reduced diphosphopyridine nucleotide (DPNH) (16). This inhibitor by DPNH was originally studied at the optimum *pH* of the enzyme, *pH* 8.7 (16). Isselbacher and Krane have shown that addition of ethyl alcohol to liver homogenate in-

creases the DPNH levels and inhibits 4-epimerase (18). They point out that the well-known decreases in galactose tolerance accompanying intake of alcohol probably can be ascribed to this mechanism.

The activity of epimerase at *pH* 7.0 is about 25 percent of that tested at *pH* 8.7 (18). *However, the inhibition by DPNH is much more severe at the acid pH.* The ratio of DPNH to DPN, rather than the absolute amounts of pyridine nucleotide, seems to be decisive with regard to the inhibition (18). In our recent studies (18), we have found that if the total DPN contains only 2.5 percent reduced DPN, the mammalian epimerase is inhibited about 67 percent at *pH* 7.0; at *pH* 8.7, however, inhibition is barely detectable (less than 20 percent). At *pH* 7.0, a steady-state level of 33 percent DPNH would only leave about 1 percent of the epimerase activity that can be measured at *pH* 8.7. Since tumors are apt to produce smaller or larger amounts of acid, depending on the supply of glucose, and since the aerobic glycolysis brings about high DPNH/DPN ratios (approaching, in some cases, perhaps 40 or 50 percent), one could almost predict that the epimerase activities in intact tumor cells would be very small, perhaps smaller than that of transferase. Based on activities determined in strain L and HeLa cells (18), this seems, indeed, to be the case, as will appear from table 1.

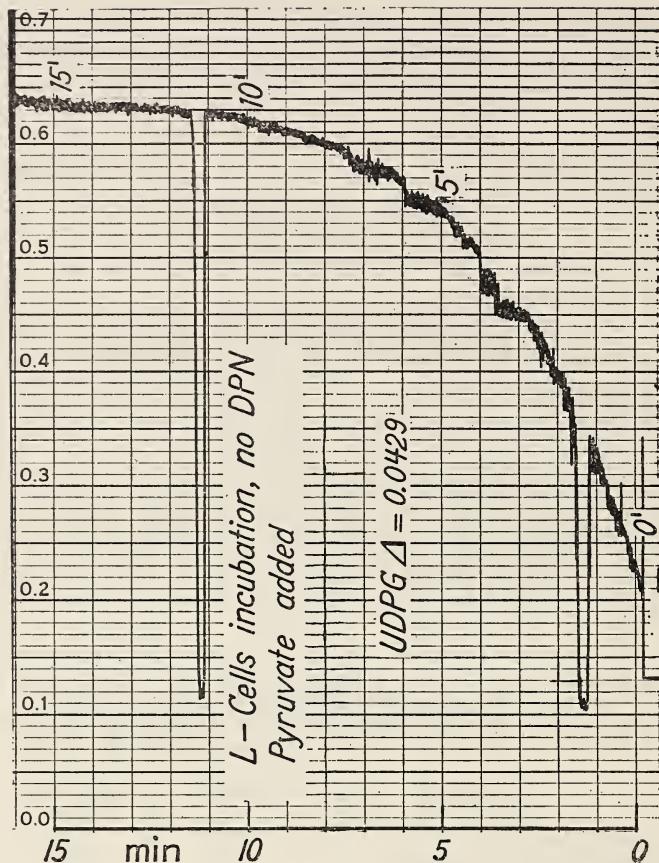
TABLE 1.—Approximate activities of galactose enzymes in broken cell preparations at 37°, *pH* 7.0, and at DPNH/DPN ratio of one third

Compounds	mμmoles per 10 ⁶ cells per hour	
	L cells	HeLa cells
Gal kinase	150	100
Gal-1-P uridyl transferase	20	30
UDP Gal 4-epimerase	2	4

At *pH* 7.5, the corresponding epimerase activities would still not amount to more than about a doubling of the above value, *i.e.*, per 10⁶ cells per hour, 4 mμmoles for L cells and 8 mμmoles for HeLa cells, dwindling amounts on the limit of present techniques of observation. The absolute amount of pyridine nucleotide may also play a role, at least if the levels are very low, a factor that may further reduce the epimerase activity.

Homogenates of mammary carcinoma or of Ehrlich ascites tumors, if incubated with glucose and uridine diphosphate galactose (UDP-Gal) for 30 to 90 minutes, show no trace of epimerase activity. This is not due to lack of or destruction of DPN, because further addition of pyruvate will bring about considerable epimerase activity, whereas nicotinamide has no effect. Broken preparations of L cells show some epimerase activity at *pH* 7.5, even without extra addition of DPN; addition of pyruvate likewise stimulates epimerase activity (text-figs. 1 and 2).

Many of our observations are at variance with those of Maio and Rickenberg (7) and Ebner *et al.* (8) who are unable to detect epimerase

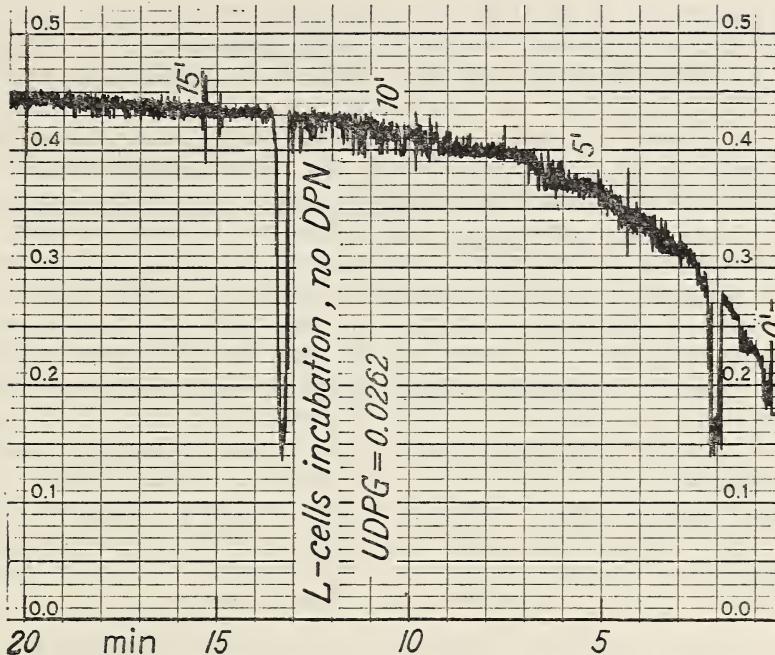


TEXT-FIGURE 1.—Enzymatic determination of UDPG formed from UDP-Gal by 1 hour incubation at 37° with 12×10^6 broken L cells, 0.23 μ mole UDP-Gal, 17 μ moles glucose, glycyl-glycine 0.1 M, pH 7.5; total volume 0.35 ml. Protein-free filtrate analyzed with UDPG dehydrogenase (Sigma) and DPN. Cary double-beam spectrophotometer, sensitive slidewire. Wavelengths: 1) "reduction band" at 340 $m\mu$; 2) isobestic point at 400 $m\mu$ ("spikes"). Amount of DPNH formed in analytical run, 2 μ moles corresponding to 1 μ mole UDP glucuronic acid formed. Epimerase activity of entire L cell digest: 40 μ moles per hour per 12×10^6 cells or: 3.3 μ moles UDPG formed per hour per 10^8 cells.

activity in broken L cell preparations, though they presumably operate at the optimum pH and add DPN.

INTERFERENCE WITH EPIMERASE ACTIVITY IN INTACT TUMOR CELLS

We have developed a technique (13) that permits us to determine quantitatively the approximate activities of the galactose enzyme in intact tumor cells, tissue cultures (L and HeLa cells), and some normal



TEXT-FIGURE 2.—Same setup as in text-figure 1, except that 5 μ moles pyruvate were added to the L cell preparation during the digestion period. Analysis of the filtrate showed that epimerase activity of L cell digest was: 6.7 μ moles UDPG formed per hour per 10^6 cells. Pyruvate alone did not reduce DPN in the indicator system. (Courtesy Biochem Z, Springer-Verlag, Berlin.)

cells of which growing and lactating mammary glandular cells are particularly proper controls. The method, employing C^{14} -labeled galactose, is only applicable if galactokinase is relatively active and transferase operates at low activity, *i.e.*, at a rate no more than about 10 percent of galactokinase, which usually would correspond to the order of magnitude of 1 to 5 $m\mu$ moles per mg protein per hour. In this way, an exposure of the tissue for 20 to 60 minutes to galactose- C^{14} will not "smear" the radioactivity farther than glucose-6-phosphate; in most cases, it does not even go beyond UDPG and glucose-1-phosphate on account of a severe inhibition of phosphoglucomutase by the accumulated Gal-1-P (13).

The technique has demonstrated a residual but minute activity of epimerase; epimerase is rate-limiting in spite of the very low transferase activities (*see* table 2). In normal cells, this technique is apt to underestimate the epimerase activity by a factor of 10 or more, since it is difficult to reduce the time of exposure to galactose below 5 minutes in the intact animal. The rate by which the UDP hexose "pool" incorporates radioactive Gal-1-P in an Ehrlich ascites tumor is very low, at the most of the order of 0.5 $m\mu$ mole per 10^6 cells per hour (13). Yet, this minute radioactive UDP hexose pool does not manage to distribute the radioactivity according to the Leloir equilibrium. If the epimerase were not

rate-limiting, the UDP-Gal/UDPG ratio should be about 0.3 to 0.4. In the Ehrlich ascites tumor, however, the radioactivity distribution between the two UDP hexoses after 30 minutes' incubation comes out as a UDP-Gal/UDPG ratio of 5. In other words, within this span of time, the conversion from UDP-Gal to UDPG has only managed to proceed about 10 percent of the way to equilibrium (18). The epimerase activity is, therefore, not significantly higher than 10 percent that of transferase and could not amount to more than 0.05 μmole per 10^6 cells per hour. In no tumor so far assayed (Ehrlich ascites tumor, rat mammary carcinoma, HeLa cells, and the partially potentially malignant L cells) did we ever find a case in which the epimerase was not rate-limiting. This seems especially important on account of the very low transferase activities found in the same instances, which means that the *absolute* epimerase activities are extremely low. This seems particularly significant in view of the relatively high growth rates of these cells. This may create a disproportion, especially if galactose-containing polysaccharides (like blood groups, for instance) have a significant turnover. The latter, combined with rapid growth, lack of a supply of exogenous galactose, and obliteration of the glucose-galactose interconversion, may make the cell incapable of keeping up patterns like antigenic mosaics.

TABLE 2.—Approximate activities of galactose enzymes in intact cells

Cell types	m μ moles per 10^6 cells per hour	
	Transferase	Epimerase
Lactating mammary gland (mouse)	4.5	$>>5^*$
Ehrlich ascites tumor (mouse)	~ 0.5	$\lesssim 0.1$
Mammary carcinoma (rat)	<0.1	<0.1
HeLa cells (homo)	~ 1.0	~ 0.2

*On account of the relatively low transferase activity, which becomes rate-limiting, the epimerase activity may be underestimated manyfold.

“EKTOBIOLOGY” OF TUMOR CELLS

“Ektobiology” was chosen as an expression of the biological characteristics of the surface of the cell. The antigenic patterns and the blood group patterns are typical ektobiological manifestations, the specificity of which depends on sequences of various sugars. Virus or phage receptor sites (and perhaps mating characters) are additional examples. It has been mentioned previously and references to the literature have likewise been made concerning the phenomenon (20) that hereditary defects in certain crucial enzymes, such as UDPG-dehydrogenase or UDP-Gal 4-epimerase, can bring about losses of several antigenic patterns. Robbins and Uchida (21) have found that the presence of a prophage in *Salmonella* can give rise to a remodeling of antigenic surface characters. The presence of glucuronic acid or galactose in the capsule or cell wall is crucial for the antigenic pattern and for the activity of virus receptor sites. *E. coli*

mutants defective in UDPG synthetase (22, 23) lose the glucose and rhamnose of the surface lipopolysaccharide (23); such mutants show normal growth rates but striking resistance to many virulent phages (24, 25).

In the literature of tumor immunogenetics, it has been emphasized (26) that the incidence of loss or weakening of blood group determinants is conspicuously frequent in malignant cells.

About 15 years ago, A. H. Sturtevant subjected the literature on cancer genetics and immunogenetics to a most interesting analysis, which as far as I know, was never published. However, in a few lectures that I happened to attend at the California Institute of Technology in early 1950, the immunological aspects of malignant growth were discussed, and from the literature available, Sturtevant posed the question whether malignancy may not be correlated in a meaningful way with various antigenic defects, especially losses of antigenic characters. The subsequent work by Gorer, Hauschka, Hoecker, and Klein (*cf* 27) furnished important direct evidence for such a correlation. The possible relation between malignancy and the runt disease has been mentioned by Tyler in an extensive review on malignancy (28). It seems a most worthwhile approach to pursue.

In a previous discussion (20), it was emphasized that a "simplification" of the ektobiology could be brought about by numerous changes in the finely balanced "network" of metabolic enzymes. However, after the realization of the crucial role of aerobic glycolysis in the interference of cellular epimerase activity, one wonders whether this factor may not be more predominant than other metabolic blocks. Exceptions are certainly known. For instance, it is important in this connection to bear in mind that certain hepatomas of malignant nature do not show aerobic glycolysis (29). If the considerations concerning the "deprecations" of ektobiological patterns, as a common feature in malignant tumors, should be applied here, one would naturally turn the attention toward the possible occurrence of a different type of block somewhere in the many biosynthetic pathways of the other peculiar sugars of the cell surface (20). Defects in the pathway of synthesis of "ektopeptides," and especially the lipides of the membrane, should also be considered in this context.

Regardless of the origin of the enzyme defect (direct hereditary enzyme defects or metabolic interference of enzymes), losses in ektobiological patterns depend, of course, on a situation in which the requirements for renewal (rate of cell division, turnover rates of constituents) exceed the capacity for synthesis. Concerning the potential role of "ektogalactose," we can say that although the rate of residual epimerase activity in the Ehrlich ascites tumor is very low, it may still be sufficiently high to permit the formation of normal ektobiological patterns.

Thanks to recent work by Wallach on the isolation of cell membranes from Ehrlich ascites tumor cells (30), it should be possible to test some of the ideas advanced here on closely related benign and malignant tumors. A quantitative determination of glucose, galactose, and sialic

acid with cytolipides before and after exposure of cells (for instance, benign and malignant ascites tumor cells) might be informative.

A biochemical and immunochemical analysis of the ektopolysaccharides of mammalian cells, and especially tumor cells, will have to take cognizance of the fact that defects in one type of polysaccharide may not be reflected to the same degree in another type. This is illustrated well by some recent observations on UDPG-defective *E. coli* mutants (table 3). The mutants have a hereditary defect of the UDPG synthetase-UDPG pyrophosphorylase. One lipopolysaccharide with glucosamine, heptose, phosphate, and glucose as the major components has lost the glucose component [less than 3% of the normal content (31)]. Another complex heteropolysaccharide with glucose and glucosamine as the main components (30-33) has retained almost 30 percent of its glucose (31). The biochemical background for this phenomenon remains to be clarified. Perhaps glucosyl nucleotides other than UDPG contribute to the synthesis of the second polysaccharide. However, it is interesting that the biology of this mutant is changed, *e.g.*, it can no longer become infected with phage P_1 (25), presumably due to a loss of phage receptor sites for this phage.

TABLE 3.—Glucose content of *E. coli* K-12 polysaccharide*

	UDPG-positive (%)	UDPG-defective (%)
Lipopolysaccharide, heptose type	11.3	0.3
Other polysaccharides	17.5	4.5
Susceptibility to phage P_1	+	0†

*From Mayer *et al.* (31).

†From Adler (25).

Dr. Wu and I have recently found that slime molds which need bacteria for growth and differentiation can grow on the UDPG-defective mutants, but that their differentiation is greatly delayed or completely arrested even before the aggregation stage.

Lactose is a constituent of cytolipin H and has been found in various tumors (34) and also in membranes of L cells (35). In some recent unpublished studies by Donald Wallach and myself, we have by means of the highly sensitive thin-layer chromatography detected galactose in membranes from Ehrlich ascites tumors [prepared according to Wallach (30)]. It is known that sialic acid is present too (30).

Although the amounts of the sugars are small in these rapidly growing cells, and can best be detected by thin-layer chromatography (36), it may nevertheless pose a problem in connection with the epimerase defect desired. Is it possible, if we carry the analogy with the *E. coli* polysaccharides a little further, that other ektopolysaccharides of the ascites tumor may reflect the defect in UDP-Gal synthesis more strikingly? The isoantigens described by Gorer (27) and Hoecker (37) would be of special interest. Needless to say, the lipoid of the cell membranes deserves considerable attention as well.

MALIGNANCY AND EKTOBIOLOGICAL PATTERNS

In a discussion of the biological control of cancer, Burnet (38, 39) expressed the view that "the only legitimate way to discuss its relation to general biology is to use the phenomenon of cancer . . . to throw light on the nature of normal controls in the organism." Burnet's selfmarker hypothesis, to some extent an immunological approach, is influenced by the discoveries of Medawar and his group on transplantation tolerance and by H. N. Green's ideas about losses of "selfmarkers" in the development of tumors from chemical carcinogens (40). Green's hypothesis was greatly strengthened by Weiler's elegant experimental observations (41). Immunological ideas have, as mentioned previously, been invoked in the case of transplantable tumors, and newer observations, especially on runt disease, have encouraged these views (28, 42, 43). *Changes in enzyme patterns of tumors might well be of genuine relevance to these concepts insofar as they affect the biosynthesis of immunologically expressive macromolecules.* Therefore, the immunological approach should not become unduly separated from an enzymological approach.

It is not intended, in the present paper, to imply that an imbalance in the ektobiological pattern is the main factor in tumor genesis. As emphasized in a previous discussion (20), and exposed so well by Dr. Luria's discussion (44), genetic factors (episomal factors, mutations) and proliferation (hormonal factors, etc.) are prerequisites for development of tumors. *Superimposed* on this, however, one can question whether the special capacity for invasive growth and the ability to kill the host organism, displayed by many seemingly unobtrusive tumors, do not require changes in the ektobiological pattern related to that observed in the runt disease. It is at the onset of this terminal change toward malignancy that the intracellular kinetics of epimerase is being considered as a crucial factor. In a variety of other malignant tumors, the existence of another metabolic block must be considered as the selective factor for the tumor against the host. This could affect the pathway of synthesis of some of the other "peculiar" sugars or the search might focus on the defects in the pathway of synthesis of the lipide component of the cytolipins or the histocompatibility factors (45-47).

RESUMEN

Cuatro tipos de células de mamíferos con potencialidades malignas muestran una grave interferencia de la capacidad de convertir glucosa a galactosa ("freno" epimerasa). Se presume que esto se debe solamente a la glicolisis anaeróbica que alcanza altos niveles en estos tipos de células y eleva la concentración de hidrogeniones así como también los niveles de nucleótidos piridina reducida, alteraciones que son conocidas por interferir seriamente la actividad de la epimerasa. La capacidad para convertir la glucosa y sus metabolitos en los compuestos complejos de galactosa de la superficie puede volverse insuficiente en las células en crecimiento rápido si se impone una seria barrera a la actividad de la epimerasa. Un "adelgazamiento" significativo de los compuestos galactosil de la superficie ("ectopolisacaridos") puede tener un efecto desencade-

nante sobre el crecimiento neoplásico contra la reacción del huésped. Se sabe por el estudio de los microorganismos que defectos de las enzimas envueltas en la biosíntesis de "ectopolisacaridos" específicos permiten el crecimiento pero alteran la "ectobiología" (pérdida de los antígenos de superficie, pérdida de los sitios receptores de virus). Los estudios de modelos de enzimas en las células neoplásicas mantienen su interés. Los defectos ó alteraciones de los modelos de enzimas en las vías de biosíntesis que conducen a la formación de "ectopolisacaridos" defectuosos puede ser de incumbencia especial para el problema de crecimiento neoplásico maligno como se ve a la luz del equilibrio "injerto contra huésped."

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DISCUSSION

Prescott: What are the effects of feeding the different bacterial mutants to slime molds? Specifically, if inhibitors are involved in the loss of epimerase activity or activity of other pertinent enzymes in the bacterium, is it possible that such inhibitors might be effective against epimerase, etc., of the slime mold after ingestion of the bacteria?

Kalckar: The whole problem arose from some considerations in which we had planned to study the effect on the ameba by bacterial mutants having defects in specific metabolic pathways involved in the biosynthesis and maintenance of chemical patterns of the bacterial surface.

The ability of the slime mold ameba to utilize phagocytosis of the various mutants was not affected, nor was the growth of the ameba. However, the ability to aggregate was markedly delayed in those bacterial mutants which were defective not only in galactosyl donors, but also in glucosyl donors. It is not clear at the present time whether this effect is due to the lack of something, or as you suggested, to the accumulation of inhibitors of slime mold differentiation. Dr. Henry Wu and I have been looking into the latter possibility. We have, for instance, found that the effect of the mutant is abolished after autoclaving.

Advances in the Ultrastructure of the Nucleus and Chromosomes¹

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SUMMARY

Some recent advances on the ultrastructure of the nucleus and chromosomes are summarized. This study is of particular importance in view of the evidences that genic information is contained within nucleoprotein macromolecules. The differences between the ultrastructure of the nucleus and cytoplasm are emphasized. The nuclear envelope is a cytoplasmic derivative of the vacuolar system. The presence of pores is described and their nature discussed. Recent work on the electrochemical properties of the nuclear envelope are mentioned in connection with the significance of pores. The ultrastructure of the nucleolus is described together with cytochemical studies that point toward a relation between nucleolar and ribosomal ribonucleic acid (RNA). According to this concept the nucleolar organizer of the chromosome would be the site or locus primarily devoted to the large-scale production of cytoplasmic ribosomes, and the presence of large nucleoli in rapidly synthesizing cells is explained

by the large number of copies of ribosomal RNA needed. Studies on the submicroscopic structure of chromatin and chromosomes together with cytochemical studies with electron stains, the action of enzymes, and autoradiography at the electron microscope level are mentioned. The possible macromolecular organization of chromosomes is discussed in view of the mechanism of replication of the deoxyribonucleic acid (DNA) molecule. This is now well understood in lower organisms, particularly in certain bacteria in which a circular single two-stranded DNA molecule constitutes the chromosome, but in higher cells only some hypothetical models with the inclusion of intermediary linkers have been proposed. The so-called synaptinemal complex of meiotic chromosomes is interpreted on these lines. The basic macromolecular organization of chromatin and chromosomes is that of microfibrils which can go down to the single nucleoprotein molecule.—Nat Cancer Inst Monogr 14: 33-55, 1964.

FOR MANY years the work of cytologists and cytogeneticists with the light microscope has been dedicated mainly to the structure of the interphasic nucleus and to the important problems of chromosomal morphology related to the linear array, reduplication, mutation, and function of genes. Details have been recognized in the shape and structure of chromosomes

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such as the presence of chromomeres, centromeres, secondary constrictions, satellites, and nucleolar organizers, all of which are very important in identifying individual chromosomes within a karyotype.

This study led also to the recognition of the chromonema as the filament that constitutes the bulk of the chromosome and which undergoes a complex cycle of coiling during mitosis and meiosis. However, the limited resolving power of the light microscope made very difficult any further analysis of the chromosomal structure. This shortcoming gave rise to many speculations and points of disagreement, such as the presence or absence of an amorphous matrix between the coils of the chromonema or of a pellicle surrounding each chromosome.

The introduction of modern methods of analytic cytology, and particularly the use of the electron microscope, have led to definite progress and from the very beginning disproved the hypothesis of a chromosomal matrix and pellicle.

The high resolution achieved on fixed and sectioned material offers the possibility of visualization within the chromosome of the nucleoproteins and other macromolecular components that can be demonstrated by biochemical methods. Genetically, the knowledge of the fine structure of chromosomes is of particular importance since evidence has identified the gene with the macromolecules of nucleoprotein. In fact, in simpler genetic systems, such as those of bacteriophages and some bacteria, it can be demonstrated that the genetic material can be divided down to its ultimate molecular structure.

SUBMICROSCOPIC STRUCTURE OF THE NUCLEAR ENVELOPE

Under the electron microscope the differences between the cytoplasm and the nucleus are striking. The cytoplasm is traversed by a very complex system of membranes, which includes the so-called endoplasmic reticulum, the Golgi complex, mitochondria, and other structures, and which constitutes a large part of its mass, while the nucleus, with exception of the nuclear envelope, has no membranous structures. As shown later, only a fine filamentous material and macromolecular granules form the bulk of the nuclear substance (fig. 1).

When one considers the ultrastructure of the nucleus, it is necessary to differentiate the nuclear envelope or membrane from the rest of the nuclear mass. One of the most interesting conclusions deducted through electron microscopy is that the nuclear envelope is a dependence of the cytoplasmic vacuolar system. This is demonstrated not only by the continuities observed at many points, but also by the study of these structures in different cell stages and phylogenetic follow-up. Most bacteria lack internal membranes and the nucleoid is devoid of an envelope; in mycobacteria a few cytoplasmic membranes appear but not around the nucleus (1). Only in fungi and in higher organisms does a definite nuclear envelope appear (2).

The early electron microscope studies were carried out on isolated nuclear membranes of amphibian oocytes in which an outer porous layer was recognized (3). The pores are about 400 Å in diameter with a regular disposition and an interporc distance of 1000 Å. Several authors made similar observations in oocytes, amoebae, and in giant polytenic nuclei of insects (4). In sea urchin oocytes, there are 40 to 80 pores per μ^2 which are covered by a single membrane and enclosed by a cylindrical wall that has been called *annulus* (5-7) and also *pore complex* (8). In amoebae a system of closely packed, hexagonal prisms has been observed at the pores (9). Watson (4, 8) has extensively studied this subject in a variety of mammalian cells, and described real gaps and breaks of 400 to 700 Å through the two membranes. According to Watson, at these pores the nucleoplasm is exposed freely to the cytoplasm, and there is no visible membrane between them. At the edges of the pores the two membranes of the nuclear envelope are in continuity. Calculations based on a pore diameter of 500 Å indicate that approximately 10 percent of the nuclear surface is exposed, whereas the rest has only an indirect contact through the membranes. It has been suggested that large molecules may pass through the membranes (4).

This concept recently has been contradicted by the very ingenious experiments of Löwenstein and Kanno (10), who investigated the electro-chemical properties of the nuclear membrane with microelectrodes and differentiated two kinds of nuclear membranes.

In giant nuclei of salivary glands of *Drosophila* they found that as the electrode is advanced from the exterior there is first an abrupt change in potential at the plasma membrane (-12 mv); then upon entering the nucleus there is another drop in negative potential corresponding to the nuclear membrane (-13 mv). At this surface there is also an electrical resistance which, though much smaller than that of the cell membrane, is large enough to indicate that the nuclear membrane must be a formidable diffusion barrier even for ions as small as K^+ , Na^+ or Cl^- . These results pose some doubts about such a continuous and free interchange between nucleus and cytoplasm through the pores. Löwenstein and Kanno conclude that the nuclear membrane must be an ion barrier and that pores are either not freely communicating fenestrations or the material within the pore complex is very resistant.

The other type of nuclear membrane is that found in oocyte nuclei, in which the resistance is so low that it cannot be distinguished from that of the cytoplasm and there is no detectable membrane potential. Here the interchange between nucleus and cytoplasm is probably continuous.

Another interesting concept derived from electron microscopy is that the nuclear envelope is a dependence of the vacuolar system forming the endoplasmic reticulum in the cytoplasm. According to this interpretation the two membranes with the perinuclear cavity represent large, flattened cisternae apposed to the surface of the nucleus.

That the nuclear envelope is in continuity at certain points with the vacuolar system of the cell has been observed repeatedly in several cell

types. This communication opens the possibility of a direct interchange of the nucleus with the surrounding medium of the cell, even if this may be intermittent. In this aspect some observations of fat droplets absorbed by the intestinal cells, and present between the two membranes of the nuclear envelope, seem particularly convincing (11). Furthermore, in telophase the nuclear envelope is formed from the vacuolar system (12). Mirsky and Osawa (2) discuss the possibility of a direct pathway between the exterior and the nucleus and mention in support of this possibility the penetration of acridine dyes into the living nucleus without staining the cytoplasm. However, other alternative explanations for this phenomenon can be raised (2, 13, 14).

SUBMICROSCOPIC STRUCTURE OF THE NUCLEOLUS

Interest in the submicroscopic structure of the nucleolus has increased considerably in recent years because of its importance in the synthesis of protein of the cell and as a source of nuclear and cytoplasmic ribonucleic acid (RNA). All three types of RNA—transfer or soluble RNA, ribosomal RNA, and messenger RNA—have been described as forming or accumulating in the nucleolus. In addition there is evidence of the possible nucleolar elaboration of the ribosomal protein. Furthermore, the RNA-methylase, an enzyme that transfers methyl groups on the RNA purines and pyrimidines at the polynucleotide level, forming bases which are characteristics of transfer RNA, has recently been located in the nucleolus (15).

The possible relationship between the nucleolus and cytoplasmic RNA was first suggested by Caspersson in 1939 and has been substantiated by the most recent investigations. It has been found that the RNA of the different parts of the cell have a distinctive base composition (16, 17), and that the RNA of the nucleolus behaves differently from that of the cytoplasm or from the chromosomal RNA with regard to the uptake of different labeled nucleosides and to the action of inhibitors of the RNA synthesis such as actinomycin (18, 19).

Much evidence indicates that the major portion of the cytoplasmic or ribosomal RNA is derived from the RNA in the nucleolus. In addition, the extranucleolar or chromosomal RNA appears to be synthesized independently from nucleolar RNA.

In a combined cytochemical study with different labeled RNA precursors, such as cytidine and uridine, and extraction and ultracentrifugation of the different RNA moieties in normal and actinomycin-treated dividing cells, Perry (20) has recently concluded that both the messenger and the transfer of soluble RNA are produced by the chromosomes. Yet, the nucleolus produces a rather heavy RNA which is transformed by an unknown process into the 28 and 18S RNA found in cytoplasmic ribosomes (see Perry, this Symposium).

According to this view [see also (21, 22)] the nucleolar organizer of the chromosome would be the site or locus primarily devoted to the large-scale production of cytoplasmic ribosomes. The presence of large nucleoli in rapidly synthesizing cells is thus explained by the many copies of ribosomal RNA needed.

The electron microscope has revealed a definite submicroscopic organization within the nucleolus (fig. 2). In some cells an irregular fibrillar structure reminiscent of the nucleolonema of Estable and Sotelo (23) can be observed (24, 25), but in others the structure is compact and relatively homogeneous. Here it appears as a dense mass constituted of a tight aggregation of round particles of about 150 Å.

The origin and evolution of the nucleolus during mitosis vary in different cell types, but there is some evidence that in telophase the nucleolar substance may originate from all the chromosomes by the fusion of small "prenucleolar" bodies which are collected in relationship with the so-called nucleolar organizer. The macromolecular particulate component is constant in all nucleoli having a compact or a more or less open or nucleolonemic structure. Sometimes surrounding the main mass of the nucleolus are smaller bodies having the same structure; these can be interpreted as nucleolar material derived by fragmentation of the outer portion of the nucleolus. Also, in the nuclear sap, between the chromosomes, there are dense particles with dimensions similar to those found in the nucleolus (26).

These dense particles may be nuclear ribosomes, but the identity with cytoplasmic ribosomes cannot be assured. In a recent study using different staining methods and enzymic treatment Marianozzi (27) has recognized in nucleoli of pancreatic cells two types of granular structures: one corresponding in size to that of ribosomes (150 to 250 Å) and another formed by smaller grains (50 to 100 Å), both of which are attacked by ribonuclease. The smaller granules are probably precursors of the larger ones and appear embedded within a protein matrix. In a recent paper Lafontaine and Chouinard (28) have followed the nucleolar cycle at both the light microscope and electron microscope level.

SUBMICROSCOPIC STRUCTURE OF THE CHROMATIN AND CHROMOSOMES

Early attempts to study the chromosomal components under the electron microscope consisted of observations of isolated lampbrush chromosomes of amphibian oocytes and nuclear fragments. There was considerable difficulty in the use of fixing, embedding, and sectioning techniques, which have been so productive in recognizing the ultrastructure of the cytoplasm, when applied to the study of the nucleus. Only after 1955, because of some improvements in fixation, was it possible to recognize a definite macromolecular structure in chromosomes (26).

These technical difficulties arise partly from inadequacies of available preparative methods, from the fineness of the macromolecular organiza-

tion of chromatin, and the complex tridimensional array of the nucleoprotein constituents, which make it difficult to interpret electron micrographs of thin sections of the nucleus.

Nucleic acids do not bind osmium tetroxide (29) and, therefore, protein probably contributes more to the image of the nucleus. De Robertis (26) found that adding Ca^{++} to the osmium fixative improved the observation of the microfibrillar structure of chromosomes. This was probably related to the fact that removal of Ca^{++} by chelating agents in a medium of low ionic strength produces dispersion of nucleoprotein from the nucleus (30, 31). More recently the use of other metal-containing cations, such as indium trichloride, uranyl acetate, and lanthanum nitrate, have been proposed for the preservation of chromatin.

According to Watson (32) these cations preserve deoxyribonucleic acid (DNA) in solution while osmium tetroxide has no effect. In the intact nucleus, however, there is no improvement of fixation, indicating that the protein is probably more important in the preservation of the nucleoprotein structure.

If the *pH* and concentration are controlled, uranyl ions have a high specificity as electron stains for structures containing nucleic acids. Zobel and Beer (33) found an association constant 800 times larger for a DNA-uranyl complex than for a protein-uranyl complex. Experiments on model systems or on fixed cells from which the osmium had been removed showed that at *pH* 3.5 and at concentrations 1×10^{-5} to 10^{-4} M uranyl acetate is specific for nucleic acids (34). Recently bismuth has been proposed as an electron stain for nucleic acid because of its reaction with inorganic phosphate (35).

Another recent approach to the ultrastructural cytochemistry of the nucleus is the use of digestion by different specific enzymes after fixation in formaldehyde and embedding of the tissue in water-soluble media (36). The action of ribonuclease, deoxyribonuclease, pepsin, trypsin, and other hydrolytic enzymes on the ultrastructure of chromosomes, the nucleolus, ribosomes, and other structures can be investigated. With pepsin the nucleolar substance is selectively attacked. Trypsin causes complete digestion of ribosomes in the cytoplasm and the nucleolus. With ribonuclease the nucleus appears practically unchanged, the nucleolus appears less dense, and the RNA-containing granules of the nuclear sap and the cytoplasmic ribosomes disappear. Deoxyribonuclease digests chromatin, but the nucleolus and the interchromosomal granules remain intact (36).

Recently autoradiographic techniques have been used with the electron microscope to study the fine structure of the nucleus and chromosomes as related to the metabolic activity of nucleic acids. Hay and Revel (37), studying regenerating limbs of salamander injected with tritiated thymidine, have followed different stages of the synthesis of DNA in the microfibrillar component of chromosomes. The uptake of H^3 -thymidine takes place during the period of active synthesis of DNA in a portion of the interphasic stage, and the grains of reduced silver are found only in those nuclei that have undergone such a stage of synthesis and not in the others.

Furthermore, it can be shown that they are located in the chromosomal regions and not in the nuclear sap or on the cytoplasm. The resolution achieved by this technique is of about 0.2μ . The dense parts of chromatin (heterochromatin) at the interphase show little or no synthesis as compared with the euchromatic regions.

ORGANIZATION OF CHROMOSOMES AND REPLICATION OF DNA

According to Taylor (38) the largest hiatus in our understanding of chromosomal reproduction and replication is at the level between the single molecules of DNA, in which are recorded the major part of genetic information, and whole chromosomes as entities defined by their microscopic organization. In molecular genetics the basic hypothesis is a consequence of the model of the DNA molecule proposed by Watson and Crick (39), which involves two complementary strands of polynucleotides. This model gave rise to the theory of replication by a template process in which each strand acts as a mold for the newly synthesized molecule. Since one of the polynucleotide chains determines the other during the synthetic period of the interphase, the mechanism can be set into action simply by the separation of each strand. This hypothesis presupposes that the nucleotides fall in phase while the two parent polynucleotide chains unwind.

The experiments of Taylor *et al.* (40) demonstrated that within a chromosome there is an atomic integrity of the two DNA subunits of which it is constituted. Meselson and Stahl (41) could demonstrate that the replication of DNA is semiconservative at a molecular level. By growing *Escherichia coli* in an N^{15} source first and then in an N^{14} source and separating the DNA synthesized in the ultracentrifuge, they could demonstrate that by the first generation an intermediary band appears. On the second generation two peaks of DNA are observed: one of N^{14} and the other intermediary. The intermediary band is composed of hybrid DNA molecules as can be shown by heating at $100^\circ C$ for 30 minutes.

Recently, Cairns (42) has elegantly demonstrated the semiconservative nature of DNA replication in *E. coli*, both at the molecular and cellular levels.

Using autoradiography and pulses of thymidine of different lengths, Cairns has been able to follow stepwise the DNA replication. The chromosome of *E. coli* consists of a single, two-strand, circular molecule of DNA, 1 to 1.5 mm long. Duplication of the daughter strands start by forming a loop (or fork) and advances at the rate of 20 to 30 μ per minute until completed, reaching the other end of the chromosome in about 30 minutes. During the whole period of duplication the two daughter strands of DNA remain joined at the distal end.

In chromosomes of higher cells the difficult problem in understanding the replication at the cellular level is that of the packing and folding of the apparently continuous DNA molecule within a single chromosome.

While in a bacteriophage of the T4 group the DNA molecule may be 50 μ long and in *E. coli*, as we mentioned, 1 to 1.5 mm long; in a large chromosome it may be 1 m long and even if it is multistranded several cm of DNA should be packed within a single chromosome.

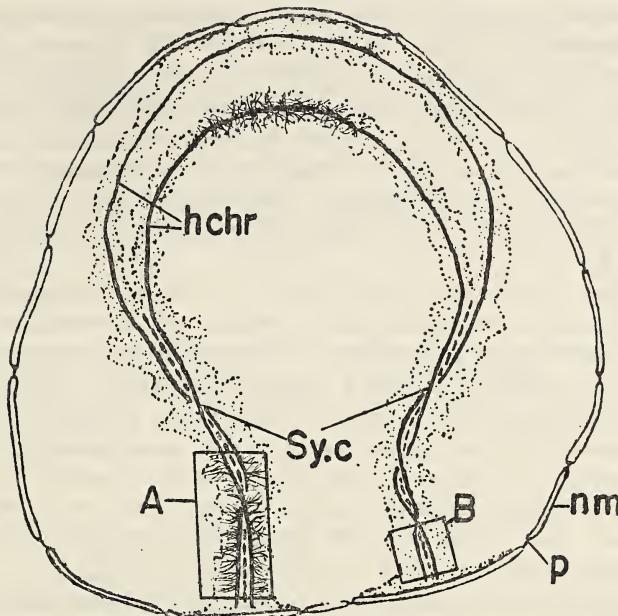
Under the light microscope the complex cycle of coiling of the chromonema can be seen in mitotic and meiotic chromosomes, but the real problem starts at a level below the resolving power of the light microscope. The model based on a single double helix extending without interruptions along a chromosome, which has been demonstrated in bacteriophages and bacteria, seems very difficult to explain the replication of the DNA in view of the long distances that would unwind and fold during this process.

Therefore Freese (43) proposed a model of DNA with many intermediary linkers. Taylor (38) has extended this hypothesis and envisioned several types of linkers, some of which could be open at operator sites for the control of the order and sequence of nucleotides. In addition to linkers joining the ends of the phosphate groups of the polynucleotide chains, other linkers would be polymers probably of polypeptide nature involved in the stabilization of the structure during replication. Each H linker would represent half a chromatid and a new set of H linkers would be formed at prophase when each chromatid is doubled. This hypothesis may be very fruitful in the future in the interpretation of the mechanism of replication at a macromolecular level, which is the particularly interesting one in electron microscopy. For the moment there is little or no morphological evidence for the existence of linkers. However, some of them may be related to the chromosomal cores described later.

SYNAPTINEMAL COMPLEX OR MEIOTIC CHROMOSOMAL CORE

In some way related to the macromolecular organization of chromosomes is the presence of a complex structure observed along the axis of meiotic chromosomes. This structure was first described in the spermatocyte of the crayfish (44) and later observed by several authors in numerous other species (45). The name "chromosomal core" that was first used implied a generalization for all chromosomes that for the moment is not warranted. Since it is thought that this axial component is related to chromosome pairing during meiosis, the more descriptive term of "synaptinemal complex" has more recently been coined [(45); text-fig. 1].

In sections observed under the electron microscope it is possible to observe the axial complex within the bivalent chromosome as two dense lines (fig. 3). Each lateral element of the synaptinemal complex is a part of a single chromosome of a homologous pair. If the pairing is not complete, the lateral parts of the axial complex may be seen to diverge. All around the axial complex is a fibrillar material making the main bulk of the chromosome. This synaptinemal structure represents an axial



TEXT-FIGURE 1.—Diagram of the synaptonemal complex according to Moses (45). Two homologous chromosomes (*hchr*) are paired in certain regions and separated in others. The pairs cross at certain points. *A* and *B* indicate a region similar to that illustrated in figure 3. Courtesy of M. J. Moses.

differentiation which appears during meiotic prophase and is probably involved in the linear pairing and interchange of homologous chromatids, whereas the main bulk of the chromosomes have the microfibrillar structure just described. These synaptonemal complexes could be related to the previously mentioned concept of linkers suggested by Taylor. These could be special differentiations of the chromosomal structure related to the pairing and interchange of fragments of chromosomes during crossing-over.

MACROMOLECULAR ORGANIZATION OF CHROMATIN AND CHROMOSOMES

At present most authors agree that chromosomes have basically a fibrillar structure beyond that observed with the optical microscope (chromatids, chromonemata). The size of the basic fibrillar unit or *microfibrils* is still not well determined. There is probably a range of the fibers of different sizes. Thus Amano *et al.* (46) mentioned: chromonema with a thickness of 1200 to 300 Å, subchromonema of 300 to 130 Å, and protochromonema of 20 to 30 Å. In lampbrush chromosomes fibrils of 500 Å were observed, each consisting of two subunits of 200 Å, and the basic structure of chromosomes has been considered to be of this order of magnitude (47). In high resolution electron micrographs of meiotic

chromosomes of the locust, a filamentous macromolecular component was described as the basic unit of structure (26). The thinnest microfibrils observed were of the order of 30 Å, and it was postulated that they represented single nucleoprotein molecules. These microfibrils varied from 30 to 170 Å with a tendency to increase in size from the early prophase to metaphase of the spermatocyte. Microfibrils are irregularly coiled and describe tight gyri and undulations. Their length cannot be determined because of the thinness of the section, but it may be several thousand Å long (fig. 4).

In the same tissue, during the development of the spermatids, a considerable change in macromolecular structure of the nuclear material occurs. In early stages microfibrils of about 50 Å are uniformly distributed within the nucleus and with a random orientation. Later, from the region of the so-called ring centriole, orientation of the microfibrils starts and progresses along with the thickening of the microfibrils (figs. 5 and 6). In later stages, they are parallel to the axis of the spermatid and about 150 Å thick. Similar observations have been made by a number of investigators. Also, in the bands of polytenic chromosomes we have seen tightly packed microfibrils with a mean diameter of 130 Å.

The most general conclusion which can be derived from these and other electron microscope observations is that, although the chromosomal structure undergoes complex cyclic rearrangement during mitosis, meiosis, and spermiogenesis, the basic unit in all stages is a filamentous, macromolecular component or microfibril, which may be as fine as a single nucleoprotein molecule. This concept is supported by observations of the more primitive forms of chromosomal material found in viruses and bacteria.

Thus the DNA contained in the "head" of bacteriophages is found in threads about 20 Å thick (48), and in nucleoids of bacteria filaments of macromolecular dimensions are observed (49). Although restricted to amoebae thus far, the observation of coiled microfibrils in Feulgen-positive areas of the nucleus are of particular interest. Pappas (50) has found helices 3000 Å long with a period of 300 to 375 Å. The filaments making up the coil are at least double and the subfilaments are paraneumically coiled.

These findings on the microfibrillar structure of chromosomes are also in agreement with the model of macromolecular organization postulated by Mazia (51), who has observed that nucleoprotein from chromosomes, sperm, and interphasic nuclei is dispersed in a medium of low ionic strength when Ca^{++} is removed. In this model the complex macromolecules of DNA protein are supposed to be held together by bridges of divalent cations.

RESUMEN

En este trabajo se resumen algunos progresos recientes respecto de la ultraestructura del núcleo y los cromosomas. Este estudio es de particular importancia en vista de

las evidencias de que la información genética este contenida en las macromoléculas de la nucleoproteína. Se destacan las diferencias entre la ultraestructura del núcleo y del citoplasma. La envoltura nuclear es un derivado citoplásmico del sistema vacuolar. Se describe la presencia de poros y se discute su naturaleza. Se mencionan estudios recientes sobre las propiedades electroquímicas de la membrana nuclear en conexión con el significado de los poros.

La ultraestructura del nucléolo es descrita junto con estudios citoquímicos que indican una relación entre el ARN nucleolar y ribosomal. De acuerdo con este concepto el organizador nucleolar del cromosoma sería el sitio donde se localiza la producción en larga escala de ribosomas citoplásmicos y la presencia de grandes nucléolos en células rápidamente sintetizantes es explicada por la gran cantidad necesaria de copias de ARN ribosomal.

Además se mencionan los estudios sobre estructura submicroscópica de cromatina y cromosomas junto con estudios citoquímicos con colorantes electrónicos, la acción de enzimas y autorradiografía al nivel del microscopio electrónico.

La posible organización macromolecular de cromosomas es discutida en vista al mecanismo de replicación de la molécula de ADN. Este es ahora bien sabido en organismos inferiores especialmente en ciertas bacterias en las cuales una molécula de DNA circular constituye el cromosoma, pero en el caso de células de seres más elevados han sido propuesto solamente algunos modelos hipotéticos con inclusión de eslabones intermediarios. El llamado complejo sinaptinémico de los cromosomas meióticos es interpretado en este sentido.

La organización macromolecular básica de la cromatina y de los cromosomas es la de las microfibrillas que pueden descender hasta la simple molécula de nucleoproteína (De Robertis, 1955-56a).

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PLATE 1

FIGURE 1.—Diagram of ultrastructure of the nucleus, showing nucleolus, daughter nucleoli, and dense particles situated in the nucleoplasm. Chromosomes are built of microfibrils: *nm*, nuclear membrane with pores; *nu*, nucleolus; *chr*, chromosomes; *dp*, dense particles of nucleolar origin. Reproduced from De Robertis (26).

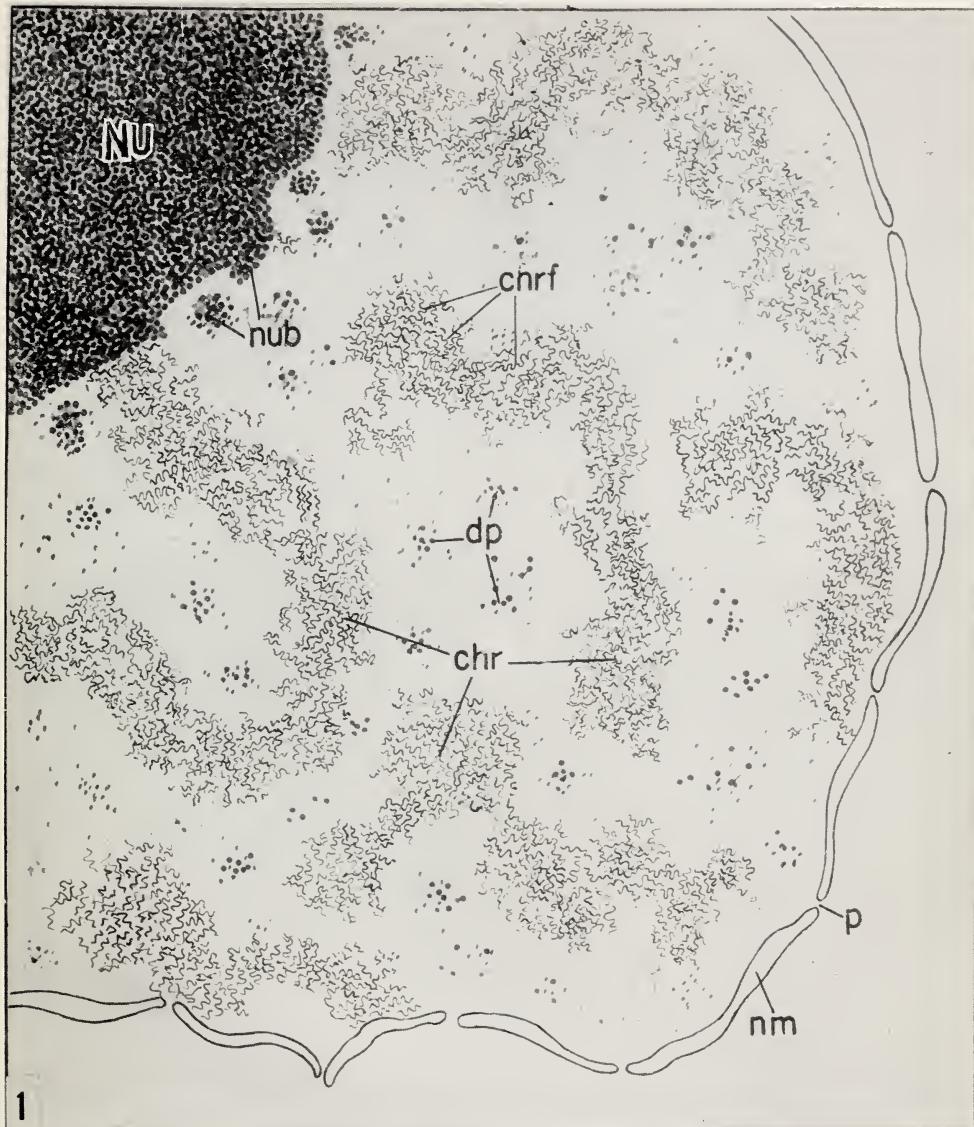
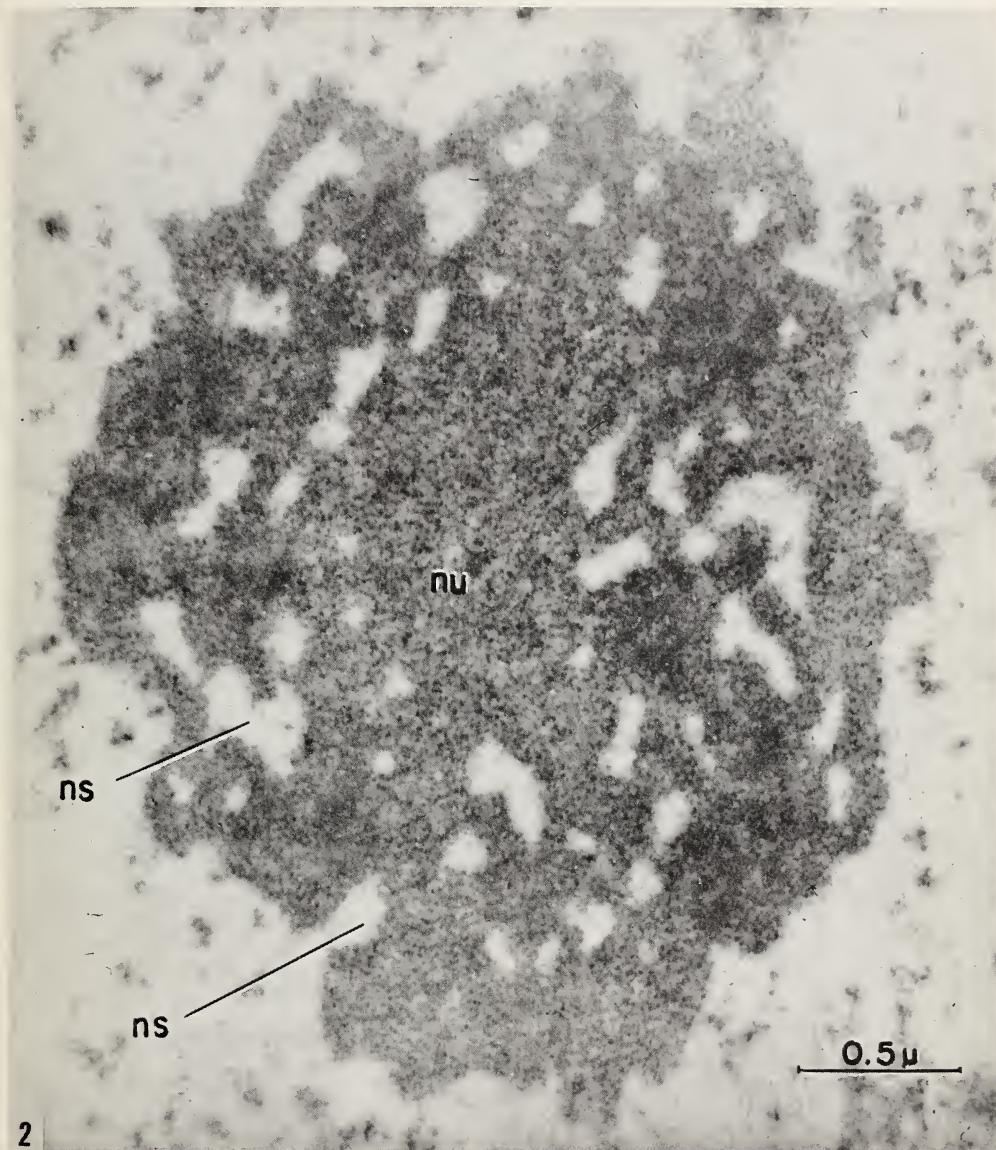


PLATE 2

FIGURE 2.—Nucleolus of a young neuroblast showing a spongelike organization in which nucleoplasm (*ns*) penetrates into mass of nucleolus (*nu*). *See* macromolecular organization of the nucleolar mass with dense particles of 150 to 250 Å. $\times 51,000$



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DE ROBERTIS

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PLATE 3

FIGURE 3.—Synaptinemal complex in a spermatocyte of rat, showing the lateral elements (*le*) and central element (*ce*), which make contact with the nuclear membrane. Outline of paired homologous chromosomes is indicated. Courtesy of Perez del Cerro from this Institute. $\times 45,000$

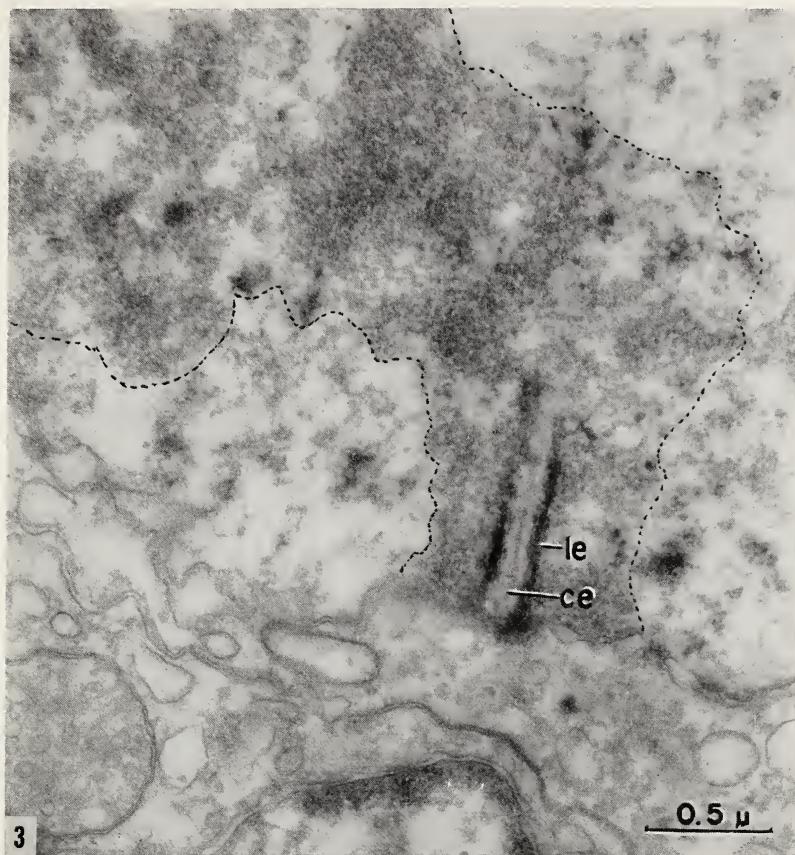
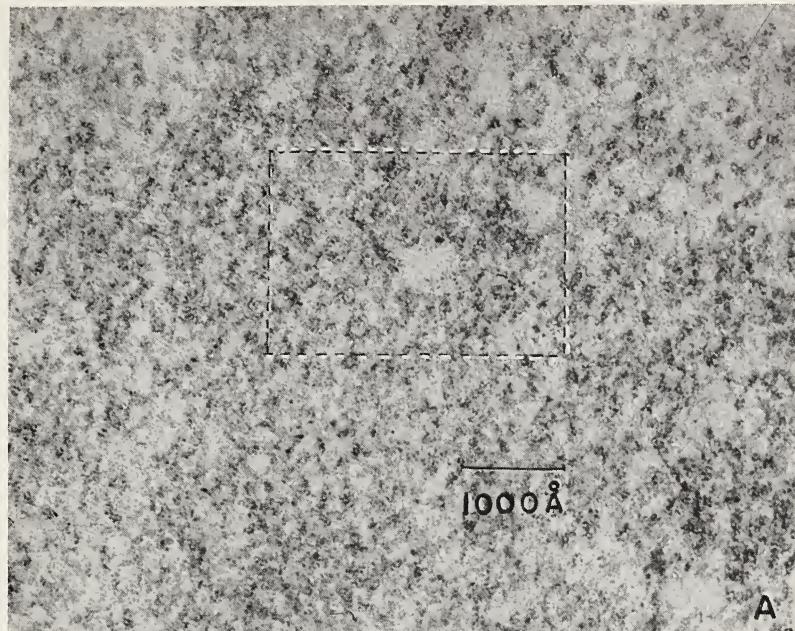
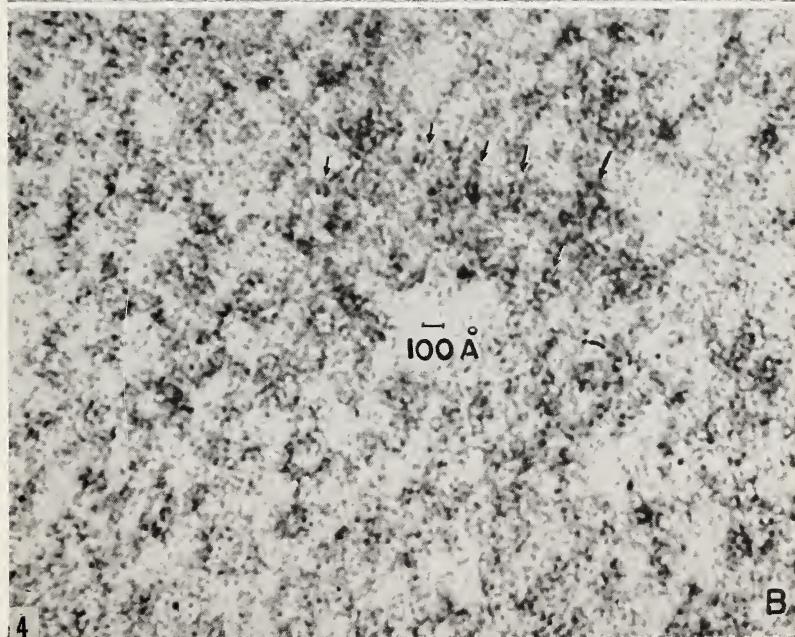


PLATE 4

FIGURE 4.—*A*: mitotic chromosome of *Allium cepa* showing fine microfibrils. $\times 180,000$ Central outlined portion is seen at higher magnification in *B*. $\times 360,000$ Arrows indicate some microfibrils 20 to 25 Å in diameter. Note the 100 Å mark. Electron micrograph made by the author of a preparation given to him by Dr. A. J. Solari.



A

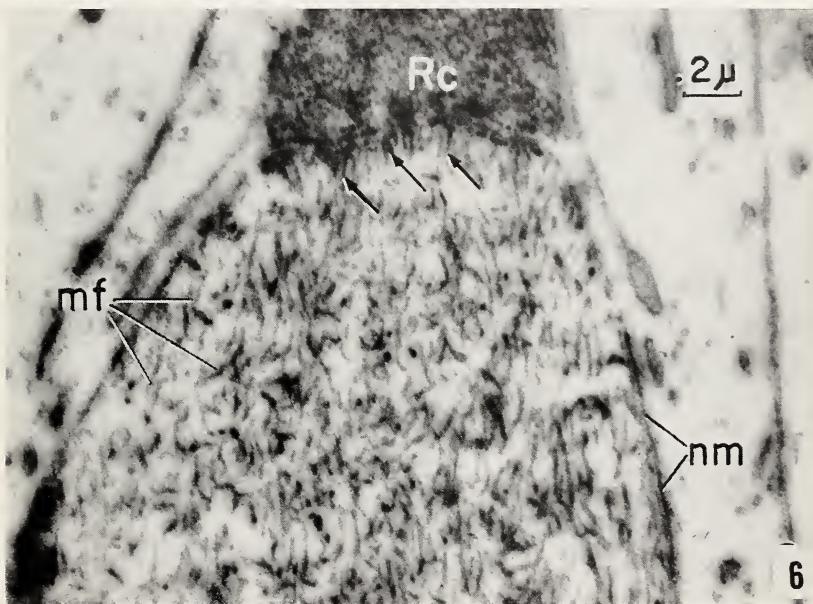
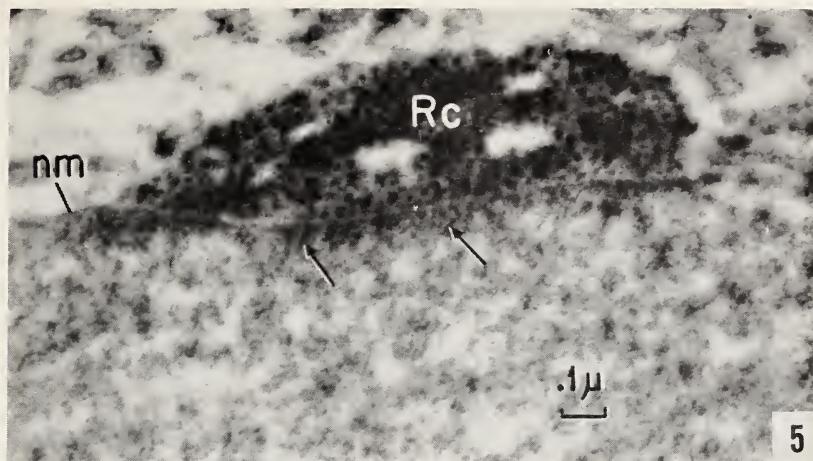


B

PLATE 5

FIGURE 5.—Region of nucleus of a young spermatid of the locust near the ring centriole (*rc*); *nm*, nuclear membrane; nucleus has a fibrillar dispersed fine structure. Microfibrils indicated by *arrows* are oriented in vicinity of *rc*. $\times 60,000$

FIGURE 6.—Later stage in development of the spermatid. Microfibrils are thicker (*mf*) and oriented in the axis of the spermatid head. $\times 40,000$ Reproduced from De Robertis (26).



Comments on the Cell Life Cycle¹

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SUMMARY

The cell life cycle is viewed as a cycle of replication and segregation of the genetic apparatus and the causal relations between these events. Control (regulated inhibition) of cell reproduction is mediated through some event of the pre-DNA synthesis period, and currently the first recognizable preparation for cell division is the initiation of DNA synthesis. The provision of adequate nucleotide pools,

the presence of DNA polymerase(s), and the conversion of DNA from a non-priming condition to a priming condition are all necessary for the initiation of DNA synthesis but are not sufficient. Some element, possibly involving the feedback of each DNA molecule upon itself—perhaps through the intermediacy of RNA and protein—must be involved in the biological regulation of DNA replication.—Nat Cancer Inst Monogr 14: 57-72, 1964.

IN ITS ORIGINAL sense, the term "stimulus" or "trigger" to cell division referred to fulfillment of some condition *immediately* before division which threw the cell into mitosis and cytokinesis. This is exemplified in earlier theories on cell division in which the attainment of a critical cell mass or a crucial ratio of cytoplasmic to nuclear volume or cell surface to cell volume was discussed as the immediate cause or trigger of cell division. In virtually all of these earlier discussions, attention was centered only on the period of division itself plus a very small fragment of time (containing the cause) immediately preceding the first recognizable sign of mitosis. A predominant attitude with respect to the remainder of the cell cycle is reflected in the terminology of the time; the period between cell divisions was called the "resting phase." Although cell physiologists recognized that the "resting phase" was a period of increase in cell size, there were no recognizable phenomena that could be directly linked to cell division, and a cell not actually engaged in division was considered to be "resting."

Specific syntheses required to bring a cell into division can now be identified far back in the interdivision interval, and certainly the term

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² Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

"resting phase" has become inappropriate. To some extent the same is true of the terms "trigger" and "stimulus" for division. Cell division is reached through a continuously integrated series of causally related biochemical activities which is initiated far back in early interphase, and in a sense a whole series of triggers is working in sequence. Perhaps the particular trigger immediately preceding—and directly leading into—division is to be considered the division trigger, but the concept has lost its former simplicity because even the selection of the criteria for marking the beginning of mitosis is arbitrary. The standard—and rather imprecise—definition of the beginning of division as the point at which chromosome condensation first becomes detectable with the light microscope is still operationally useful for marking the progress of the cell cycle, but it has little value in regard to concepts concerning the control and initiation of division. When chromosome condensation becomes visible in the light microscope, the process has already reached an advanced stage. The electron microscope might ultimately permit a more accurate, but probably no more useful, estimate of the time when chromosome condensation begins. Pushing the definition to the molecular level, the initiation of mitosis might be considered as the first coiling turn of a deoxyribonucleic acid (DNA) double helix and its associated macromolecules. The fact is, however, that although the arrival at chromosome condensation may be a reasonably definable point in the cell cycle by one criterion or another, it is still only one phenomenon in a circular chain of causally related events, most of which unfortunately are less clearly definable but no less essential parts of the cell life cycle.

The establishment of DNA synthesis as the primary event of chromosome replication and the discovery that DNA synthesis is restricted to interphase were the major factors in shifting the emphasis from cell division itself to the complete cell life cycle in which chromosome condensation and division represented just one section of events. The temporal separateness of chromosome replication (DNA synthesis) and segregation (mitosis or division) subdivides the cycle into four successive intervals: G₁, the period between the completion of division and the beginning of DNA synthesis in the first chromosome; S, the DNA replication period; G₂, the period between the end of DNA synthesis and the first cytologically recognizable sign of cell division; and D, the period of division itself (1). The S and D periods are defined by specific events but the G₁ and G₂ periods represent intervals to which it is not yet possible to ascribe any biochemical activities that are specific parts of cycle progress or continuity, and the positive basis for the existence of these two intervals is not known. S and D contain respectively the replication of the genetic apparatus and its segregation into two functional units, and the coordination of these two basic events must be mediated across the G₁ and G₂ intervals, but for the present the linking events can be little more than guessed at.

In this view of cell proliferation, the most basic events of the cycle are the replication and division of the genetic apparatus and the causal

connections between them. The various elaborate structures found in different cell types are all considered to subserve directly or indirectly the maintenance of the DNA cycle. At what level centrioles, mitotic spindles, kinetochores, etc., can be considered fundamental is less easy to decide. These highly evolved structures contribute to the functional requirements for a highly evolved genetic apparatus, and in their present form they cannot be identified with their functional counterparts in more simply organized cells such as bacteria. Virtually nothing is known about the mechanisms segregating the halves of the replicated bacterial chromosome, but whatever element or specific condition accomplishes this precise separation it is probably comparable in a fundamental biochemical sense to such structures as centrioles, kinetochores, etc.

Cytokinesis should perhaps be given separate consideration since in some systems (slime molds, some amoebae, various other syncytial systems) it is only sometimes a part of the reproductive cycle. For example, in the multinucleated amoeba, *Pelomyxa*, which normally undergoes two cytokineses (into three units) for every three nuclear replication cycles, nuclear proliferation can be maintained indefinitely without any normal cytokinesis by simply limiting the mass of the syncytium through cutting the organism in half with a needle during interphase. Cytokinesis, whether it occurs intermittently or precisely with every nuclear cycle, is in some way coordinated with nuclear division, but nuclear division is the primary event.

In some bacteria DNA synthesis may be almost continuous throughout the cycle, and the G1 and G2 periods may be virtually absent (2). Even though G1 and G2 may not then be measurable as time intervals, probably they can be considered to be present as events, in the first instance (G1) initiating a new round of DNA synthesis, and in the second (G2) operating between the completion of replication and initiation of the separation of replicated DNA into two units (nuclei). In fact, it is conceivable that the replicated portion of the bacterial chromosome may begin separation into two units before synthesis is finished in the unreplicated section. In at least one metazoan cell, the grasshopper neuroblast, DNA synthesis is continuous from telophase to prophase with no measurable G1 or G2 (3). Whether or not these two periods are discrete time intervals, however, the causal relations that coordinate DNA synthesis and nuclear division still remain to be dealt with.

In mammalian cells *in vivo* and *in vitro* the S period is typically about 7 hours, G2 lasts 1 to 3 hours, and cell division takes about 40 minutes. The period between nuclear division and the initiation of the next round of DNA synthesis (G1 period) is the most variable in duration, but ordinarily *in vitro* it lasts about 8 hours.

The G1 and G2 intervals are superficially similar. Both lack DNA synthesis and are periods of active protein and ribonucleic acid (RNA) synthesis, but their different positions with respect to DNA synthesis and nuclear division certainly mean that their compositions are basically dissimilar in certain specific and essential ways. The G2 period tends to

be of rather fixed duration (although under some situations it may be considerably prolonged) (4), and all cells finishing DNA synthesis and entering G2 always progress steadily toward cell division. When cells cease proliferative activity, they do not stop in S or G2 but go on to completion of division and come to rest in G1. This is true of mammalian cell populations *in vitro*, for example, and may be a generalization virtually without exceptions. Microorganisms brought to stationary phase by one means or another apparently cease proliferation by remaining fixed in the pre-DNA synthesis state (G1).

In fact there is some question whether G2 really exists as an interval of time at all in any cell. The earlier the beginning of prophase can be recognized, by definition the shorter the G2 interval becomes. Certainly some of what is now granted to G2 is occupied by chromosome condensation at a level below the resolution of the light microscope and is therefore a part of division (D). Conceivably all of G2 is taken up with such activity, and G2 is simply an early part of division. In such a circumstance the end of DNA synthesis may lead directly into prophase and G2 remains only as the event itself which marks the transition from replicating to condensing chromosome without occupying a measurable segment of time. Nor is it even necessary to suppose that all chromosomes arrive at G2 in synchrony. In certain mammalian cells, particular chromosomes consistently finish DNA synthesis before others (5). Therefore, chromosomes may enter G2 at different time points, some entering G2 while others are still completing S.

During the DNA cycle in cell reproduction the release of genetic information from DNA through RNA synthesis appears largely continuous, but there are two periods of the cycle, *i.e.*, during DNA synthesis and mitosis, when the configuration of DNA (or the complex of DNA with RNA polymerase) becomes altered, and RNA synthesis is affected.

Logically it seems necessary that a DNA molecule engaged in self-replication cannot serve as a template for RNA synthesis. The relatively small depression in RNA synthesis that has been observed during the period of DNA synthesis has been interpreted in this way. Since only a fraction of the DNA is undergoing replication at any one moment, however, the effect on total nuclear RNA synthesis should be expected to be small.

The hypothesis of mutual exclusiveness of RNA and DNA syntheses can be tested directly in the ciliated protozoan, *Euplotes*. The macronucleus of this ciliate has the shape of a long, narrow rod and early in interphase a bandlike structure originates at each tip of the rod. During the remaining 8 or 9 hours of interphase the two bands travel through the nucleus, meet at the center, fuse, and disappear just before nuclear division. The bands are cytological manifestations of two waves of DNA synthesis (6). Figure 1 shows an autoradiograph of an *Euplotes* nucleus isolated from a cell that had received a 20-minute exposure to H^3 -thymidine. The radioactive DNA is restricted to two very short segments of the nucleus. The short length of nucleus in figure 2 is from a cell exposed to H^3 -thymi-

dine for 2 minutes; a line of silver grains is present over the band, which is faintly visible beneath. This restriction of DNA synthesis to the narrow replication band gave an opportunity to examine the status of RNA synthesis during DNA synthesis. Nuclei were labeled for short periods with H^3 -uridine and the isolated nuclei autoradiographed. The level of H^3 -uridine incorporation was high throughout the nonreplicating parts of the nucleus but completely absent in the region of DNA synthesis (fig. 3); for a fuller discussion of this work, see (7). The experiment demonstrates that DNA does not simultaneously support both DNA and RNA syntheses. Whether the change in function from RNA synthesis to DNA synthesis and back to RNA synthesis, after replication, is brought about by a change in the molecular state of DNA, or a change in the associated polymerase, or both, cannot be decided. Electron microscope examination of the nucleus, however, has revealed striking changes in the fine structure organization of DNA in preparation for replication (8).

The RNA synthesis of interphase continues during early interphase but by late prophase the chromosomes have become highly condensed, and RNA synthesis ceases. The autoradiograph in figure 4 shows mammalian tissue culture cells that had been incubated for 15 minutes in medium with H^3 -uridine. Interphase nuclei are well labeled but mitotic stages show no RNA synthesis (9).

These are rather gross experiments which show an interruption in RNA synthesis during the cycle without regard to the type of RNA or its information content. To what extent sequential gene activation (sequential production of different informational RNA molecules) occurs over the cell cycle has not been determined, but that such sequence may participate in the regulation of the cell cycle is at least strongly suggested by the sudden synthesis of thymidine kinase immediately before or just at the beginning of DNA synthesis (10-12).

Control of DNA synthesis.—The pre-DNA synthesis or G1 condition is not only the most variable in length, it is the state in which cells usually become fixed when they cease proliferative activity. Apparently the interruption of the cell cycle is most easily achieved in G1, and it is by the inhibition or prevention of some event in this phase that control is imposed on the proliferative activity of cell populations in multicellular organisms. Once a cell escapes the G1 interval and enters DNA synthesis, it proceeds through G2 and cell division normally without the possibility for further interruption until the next G1. These facts indicate that the decision for cell division occurs at the transition from G1 to S or shortly before. Since the initiation of DNA synthesis marks a sharp change in cellular activity and is the earliest point in interphase at which division preparation can be recognized, it is beginning to receive more study from the standpoint of the control of the cell division cycle.

Several hypotheses have been proposed to explain how DNA synthesis is initiated. The mass action hypothesis that synthesis begins when the pools of deoxyribonucleoside triphosphates reach a critical level is in some way reminiscent of the classical hypothesis that cell division com-

mences when the cell reaches a critical mass. Nucleotide pools are necessary for chromosome replication, but it is almost certain that such pools do not control or regulate replication. In certain mammalian nuclei, for example, not all chromosomes begin synthesis at the same time (5), and it is difficult to understand why the nucleotide conditions adequate for one should not also be adequate for another, if such nucleotide pools triggered synthesis. It has also been suggested that the induction of DNA polymerase synthesis might be the immediately controlling event in the timing of DNA synthesis, but DNA polymerase is apparently present throughout the cell cycle including non-DNA synthesis periods (13). Therefore, it is difficult to see how this enzyme could exert primary regulation, unless the control involved in some way the association and disassociation of the polymerase with the DNA molecules (13). Finally, the initiation of synthesis has been discussed in terms of the conversion of nonprimer DNA to the primer condition. Whatever such a change means in molecular terms, certainly it must occur; but the hypothesis does not lead very far in understanding the G1 to S transition. Immediately it must be asked what causes the conversion from one state of DNA to another, and in effect one question has been replaced with another without much additional information having been added.

In summary, the presence of adequate nucleotide pools, DNA polymerase, and primer formation are all necessary for DNA synthesis, but it seems doubtful that they regulate or control its initiation. Some element, possibly involving the feedback of each DNA molecule upon itself, perhaps through the intermediacy of RNA and protein, must be involved in the regulation of replication.

A direct examination of the *biological* regulation of DNA synthesis may be possible eventually, but in the meantime more limited and less direct questions must suffice. Returning to the ciliated protozoan *Euplotes*, the initiation of DNA synthesis at the two tips of the nucleus occurs with precise synchrony. The question of whether this synchrony is mediated through some condition within the nucleus or through the cytoplasm is answered by an examination of organisms containing two macronuclei in the same cytoplasm (such binucleated cells are obtained by fortunate accidents). In this case DNA synthesis begins synchronously at the four tips of both nuclei, which demonstrates that the synchronizing influence is mediated through the cytoplasm (14). The cytoplasmic conditions could possibly be the buildup of nucleotide pools, but this seems less likely in view of the evidence that such pools are localized in the nucleus (15). *Euplotes* normally contains, in addition to the highly polyploid macronucleus, a small diploid micronucleus. The S period for the micronucleus is temporally completely separate from the S period of the macronucleus, and whatever cytoplasmic condition must be met for the synchronous initiation of DNA synthesis at the tips of the macronucleus is not sufficient for the initiation of synthesis in the micronucleus sharing the same cytoplasmic environment. Thus, while the cytoplasm may contribute in some way to DNA synthesis, the position of such synthesis within the

cell cycle appears to be governed by unique properties of the nucleus itself.

The behavior of two nuclei in *Euploites* resembles to some degree the behavior of chromosomes in a mammalian cell enclosed within one nucleus. There is some evidence that the replication of chromosomes within a single nucleus occurs according to a specific temporal pattern (5), and although all of the chromosomes may be synthesizing DNA most of the time, some begin a little later than others and some terminate earlier than others. Large parts of a few particular chromosomes are well out of step with the main chromosomal mass. While all the chromosomes may share the same total nuclear S period, each chromosome may possibly possess its own unique S period.

The possibility that the *synthesis* of some protein may be required for the *initiation* of DNA synthesis has been examined with bacterial cultures, but the results have not been completely consistent (16, 17). In the ciliated protozoan, *Tetrahymena*, the question has been examined with single cells in known stages of the cell cycle (12). The DNA synthesis period in this cell begins after completion of about 30 percent of interphase and ends at about 70 percent. If a cell is deprived of an essential amino acid (such deprivation stops all detectable protein synthesis within a few minutes) any time between cell division and the beginning of DNA synthesis, the next cell division will not be reached. If the amino acid is withheld any time after the beginning of DNA synthesis, the next cell division always occurs. From these experiments it is obvious that some amino acid dependent event essential for the completion of the cell cycle takes place at the G₁ to S transition point. The surprising finding is, however, that the amino acid deprivation between cell division and DNA synthesis does not prevent the initiation of DNA synthesis. Under this experimental condition DNA increases about 20 percent and no more. Secondly, in this 20 percent increase in DNA, the cell is unable to use H^3 -thymidine from the medium, although normally thymidine is rapidly taken up and incorporated. To explain these experiments thymidine kinase has been used as a model, although other enzymes concerned with maintenance of the deoxyribonucleotide pools are probably involved. Without the essential amino acid, a cell is unable to synthesize thymidine kinase at the transition from G₁ to S and H^3 -thymidine of the medium cannot be utilized. However, DNA synthesis begins utilizing the pre-existing pool of nucleotides and comes to a stop when these pools are exhausted (20% increase in DNA). If a cell is allowed to make the G₁ to S transition before deprivation of an amino acid, DNA synthesis goes to completion and H^3 -thymidine of the medium is utilized. The experiments strongly imply that thymidine kinase is synthesized at the very beginning of S and is destroyed (as opposed to inhibited) when S is finished. The interpretation is supported by the fact that *Tetrahymena* do not take up H^3 -thymidine into an insoluble pool during non-S parts of the cycle, although such a pool exists during the full cycle (15). The pool is apparently replenished only during S.

Experiments of this type add information about the events occurring around the point of initiation of DNA synthesis and help to outline the problem more clearly, but unfortunately they do not add in a conceptional way to an understanding of the G₁ to S transition. The experiments do indicate, however, that protein synthesis is not necessary for the *initiation of DNA synthesis*, although some new protein is needed for maintenance of DNA synthesis.

The eventual elucidation of the immediately regulating event in the initiation of DNA synthesis will shift attention to preceding events of the causal chain within the G₁ period and ultimately back to the previous cell division. The biochemical activities which form the basis for G₁ and extend through its course in some way establish the requisite conditions for DNA synthesis. These activities of G₁ should prove to be particularly interesting since it is at this stage that the proliferative capacity of a cell is regulated.

RESUMEN

El ciclo de vida celular es considerado como un ciclo de replicación y de segregación del aparato genético y de las relaciones causales entre estos eventos. El control (inhibición regulada) de la reproducción celular se hace mediante algún evento que antecede el período de síntesis del DNA, y generalmente la primera preparación visible de la división celular es la única de la síntesis del DNA. La provisión de adecuados "pools" de nucleótidos, la presencia de polimerasa(s) DNA, y la conversión del DNA de una condición no-activa a una condición activa, son todos requisitos necesarios para la iniciación de la síntesis del DNA pero no son suficientes. Algun elemento, que posiblemente comprometa el mecanismo inhibidor de la síntesis de cada molécula del DNA por sí mismo—quizás por intermedio del RNA y proteína—debe estar envuelto en la regulación biológica de la replicación del DNA.

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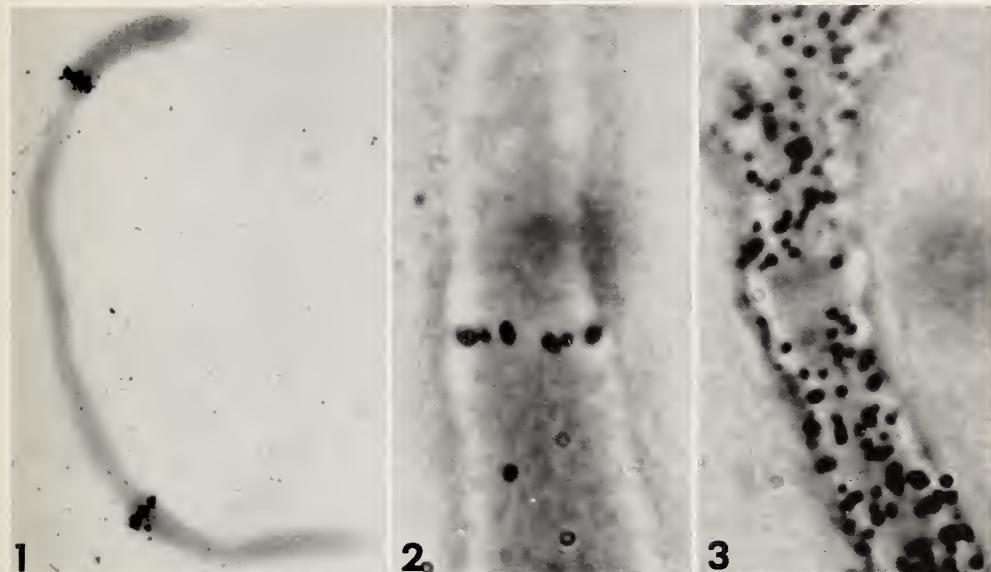
PLATES

PLATE 6

FIGURE 1.—An autoradiograph of a macronucleus isolated from an *Euplotes* incubated in H^3 -thymidine medium for 20 minutes. The heavy concentrations of silver grains cover rear zone of each band and extend for a short distance behind each band. Arrows indicate direction of movement of the waves of DNA synthesis.

FIGURE 2.—An autoradiograph of a macronucleus from an *Euplotes* incubated in H^3 -thymidine medium for 2 minutes. Single row of silver grains is over rear zone of the band.

FIGURE 3.— H^3 -uridine labeling of an *Euplotes* macronucleus for 30 minutes. Labeled RNA is present in all regions of the nucleus except the DNA replication band.

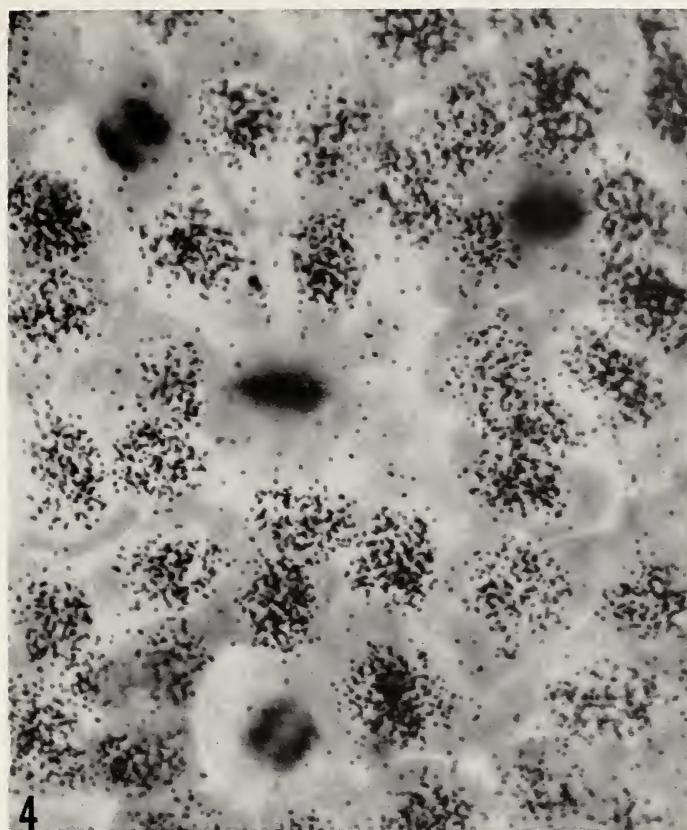


PREScott

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PLATE 7

FIGURE 4.—Autoradiograph of mammalian tissue culture cells incubated for 15 minutes in H^3 -uridine. Nuclei of interphase cells are heavily labeled but mitotic stages show no labeling.



DISCUSSION

Mazia: I would like to add to Dr. Prescott's report some general comments on the meaning of "control" in the context of DNA synthesis and cell division. In molecular biology, we are concerned with two levels of "control mechanisms": those concerned with the modulation of biochemical machinery already in existence and those concerning the production of biochemical machinery. If we are speaking of growing cell populations, as Dr. Prescott has done, we are primarily concerned with the first kind of control. All the cells are certain to divide—that is a condition provided by the experiment—and decision is not *whether* there will be DNA synthesis but *when* it will be switched on. There is all too little information about the relation between the time the S period begins and other events of the cell cycle. Cases exist where there is no G₁ period at all. For example, in the sea urchin eggs, DNA synthesis during early cleavage begins as soon as mitosis is over; that is, the minute the chromosomes are contained in a membrane (Hinegardner, Rao, and Feldman, *Exp Cell Res.* In press, confirming an earlier conclusion by Nemer). In other cases, S begins quite late in interphase, and the literature suggests that the length of G₁ is sensitive to experimental conditions. More can be said of the nature of the event by which DNA synthesis is turned on, though not in detail. It seems likely that the switch-on mechanism involves some change in the DNA, either in its structure or its associations, making DNA available as primer. Perhaps the G₁ period represents a period of mobilization of enzymes or substrates for the replication, followed by the switch-on event, but this has not been proved. But in any case, the "control" represented by the initiation of DNA synthesis is not necessarily a device for the control of cell division in differentiated systems. It would be that only if we assumed that all cells, those preparing to divide and differentiated cells not preparing to divide, synthesized the enzymes and substrates for DNA synthesis, and differed only with respect to the switch-on mechanism. We do not know much about this, but when it has been studied there seems to be a correlation between the rate of production of new cells and the amount of DNA polymerase. In the sea urchin embryo, Hinegardner and I have found a close correlation; the nuclear polymerase declines as the development proceeds and cell division slows down; admittedly this might not be a typical case. But I would like to emphasize the possibility that the second kind of "control mechanism"—not merely a decision whether DNA synthesis itself will be turned on but a decision whether the machinery for DNA synthesis will be made, in other words, a control mechanism of the type being explored by students of enzyme induction and repression—might be the important one and the one relevant to the purposes of this Symposium. Such a mechanism would not exist in a decisive way in populations of dividing cells, which by definition are cells set free from such controls. Before we can discuss the control of cell division in differentiated systems we will have to find out what level of control we are dealing with.

Prescott: I will agree also with everything you have said except for one rather trivial point; because one cannot measure G₁ and G₂ does not mean they are absent; they may be extremely short, and I think we should think of G₁ and G₂ not in terms of time periods, but in terms of events.

I forgot to mention, by the way, that the experiments with amino acid deprivation in *Tetrahymena* also show that thymidine kinase and probably other enzymes concerned with maintenance of the deoxyribonucleotide pools are destroyed when DNA synthesis is over. Such enzymes must be resynthesized at the beginning of the next DNA synthesis period. This derepression, which occurs at a precise point in the cell cycle, deserves study particularly because of its significance in relation to the control of DNA synthesis.

Court Brown: Dr. Prescott, do you believe that there are factors outside the cell also involved in the control of division? We have very good evidence that if one takes a random sample of the normal general human population and uses a blood culture technique that the proportion of aneuploid cells in culture increases with age. One

can show that in males, in which one can recognize the Y chromosome, that this is entirely explained on the basis of abnormalities of cell division involving the Y chromosome, and that in females it is entirely explained on abnormalities of division involving a medium chromosome, which we presume is the X, and quite possibly the late-replicating X. Now the effect in males appears to have a simple relationship with age, but in women the relationship seems complex. It is linear up to and about the age of the menopause, and then for the 2 decades after menopause very striking changes take place at a time when one presumes that there are changes taking place in the endocrine status of the woman. Do you think it possible that extracellular factors, such as the sex hormone levels, may influence the time of replication of DNA in the sex chromosomes?

Prescott: Whether endocrine factors are involved in nondisjunction of the X chromosome and chromosome 21 (22?) is difficult to say. More likely, misassortment at division is related to the late-replicating behavior of these chromosomes. *In vitro* work would suggest that late replication of a chromosome is unrelated to endocrine conditions.

Perry: Concerning your statement, Dr. Prescott, that in a sense everything in the cell cycle is subservient to DNA synthesis: that is, given DNA synthesis, everything else follows. I really cannot see this as a statement of absolute fact, because if you consider a cell with gene products—say N units of gene product—this cell has to form two cells, each of which has N units of gene products. Take ribosomes, for example, before a cell can divide, it has to double its ribosome content during the cell cycle. If it synthesizes DNA it still will not divide unless it also synthesizes enough ribosomes so that the next generation will have a comparable complement of machinery for protein synthesis. If this were not so, the cells would not be functional. The fact that the gene products as well as DNA have to double through the cell cycle makes the statement that everything is subservient to DNA synthesis a little too strong.

Leblond: Is your evidence that RNA is not synthesized during DNA synthesis limited to *Euplotes*? If the phenomenon is general, is it due to the fact that, perhaps because of the amount of energy available or for steric reason, the DNA strands can only do one job or the other, that is, either DNA or RNA synthesis. There might thus be a dichotomy in DNA function.

Prescott: There is a dichotomy, but I do not believe that the dichotomy is based on the point you suggested, namely, energy metabolism. We studied the macronucleus of the ciliate, *Euplotes*, in which, during the S period, less than 1 percent of the DNA is undergoing replication at any one moment. DNA synthesized is restricted to small areas called replication bands. It can be demonstrated by autoradiography that RNA synthesis takes place in all regions of the nucleus except the replication band, *i.e.*, the region of DNA synthesis.

Leblond: Does the RNA synthesis stop first?

Prescott: The replication bands move through the elongated nucleus over a period of several hours. RNA synthesis stops immediately in front of the advancing band and recommences behind the band in the region of completed DNA synthesis into that, but, in any case, this DNA is metabolically inert just as the sperm head is inert. This may be a bad analogy, but one cannot see the incorporation of precursors into RNA in this region.

Question: Are not some cells of the normal liver in the G2 phase?

Prescott: I know of no evidence on this point. Gelfant has evidence that there is a population of cells in the epithelium of skin that is moving through G2 at a very slow rate. Whether these are cells stalled in G2 is another question.

Mazia:* I believe that Gelfant has reported good evidence that the G2 populations in ear epithelium includes a group of cells that have a longer G2, but that he has excluded the idea of a population that is stalled in G2 awaiting some emergency. The latter, of course, would be a very nice adaptation.

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Upton: It was my impression, based on work in several laboratories, that a number of investigators have postulated the existence of a G0 phase during the G1 period. Evidence for this is the finding that, when partial hepatectomy is carried out, a substantial interval elapses before any of the cells in the remaining liver are observed to enter DNA synthesis, suggesting that the cells are not randomly in G1, but are actually predominately arrested in some early part of G1, which has been called the G0 phase. Whether a minority of cells is present also in G2, I do not know, but I think the bulk of the evidence is that the cells in the liver must be in early G1.

Prescott: I see no reason to call it G0; why not just call it early G1? Freshly cultured blood leukocytes take a long time to get into S, but until G0 can be defined by a particular event, I prefer to call the whole phase G1.

Luria: Dr. Jacob recently reported at Cold Spring Harbor an experiment done in collaboration with Brenner and others. Although done in bacteria, it is a very elegant illustration of the kind of mechanism that Dr. Prescott has discussed as specifically needed for initiation of DNA replication. The experiment consists of the following: In the bacterium *Escherichia coli* you can have, in addition to the main gene string or chromosome, some independently replicating accessory genetic factor, such as the sex factor. These factors can be altered so that they also contain specific groups of genes from the chromosome. For example, you may have an F-factor (sex factor) that contains the genes for the utilization of galactose and another F-factor that contains the genes for the utilization of lactose. If these F-factors are present in a cell which is galactose-negative and lactose-negative, one can follow the persistence of these extrachromosomal factors. Jacob's experiment consisted of isolating an F-factor that contains lactose genes (F-lac) and is heat-sensitive, in the sense that it replicates at 30° C and not at 42° C. So, a lac⁻ cell carrying this F-lac⁺ factor is lactose-positive at 30° C but becomes lac⁻ when growing at 42° C. If, however, the bacteria carry both the heat-sensitive F-lac⁺ factor and heat-stable F-gal (galactose) factor, they continue to grow as lac⁺ at 42° C. This proves that the F-factor that is heat-resistant produces something which is required by the other factor so its genes might be duplicated. This is the first study I know of suggesting the requirements for a specific gene product for the replication of a specific class of DNA.

Role of the Nucleolus in Ribonucleic Acid Metabolism and Other Cellular Processes^{1,2}

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SUMMARY

Nucleolar function has been shown to be indispensable in cells which are continually growing and dividing. Most likely this is related to the fact that the nucleolus is a special site for the production of ribonucleic acid (RNA). The following lines of evidence are presented to demonstrate that nucleolar RNA is an obligatory precursor of the ribosomal RNA of the cytoplasm: 1) The kinetics of the incorporation of radioactively labeled nucleosides into nucleolar and cytoplasmic RNA fractions were consistent with a precursor-product relationship. 2) Suppression of nucleolar RNA synthesis by selective irradiation of nucleoli with an ultraviolet microbeam led to a marked reduction (~70%) in the appearance of newly formed cytoplasmic RNA. 3) Treatment of cells with a low concentration of actinomycin D

selectively blocked nucleolar RNA synthesis and resulted in the complete inhibition of ribosomal RNA synthesis; however, treatment with actinomycin did not inhibit the conversion of nucleolar RNA to ribosomal RNA. Sedimentation analysis indicated that newly synthesized nucleolar RNA had a sedimentation constant of approximately 45S which is considerably larger than either of the ribosomal RNA components (18 and 32S). This fact has led us to search for the mechanism which is used by the cells to transform 45S RNA into ribosomal RNA. The transformation did not occur at 5°C and was apparently inhibited when the precursor RNA contains azaguanine. On the other hand, it did not seem to be dependent on the concomitant formation of new protein.—Nat Cancer Inst Monogr 14: 73-89, 1964.

IN THE cells of most higher organisms nucleoli exist as compact ribonucleic acid (RNA)-containing organelles intimately associated with specific chromosomal loci. In many types of rapidly proliferating cells—cells in which growth and division comprise a major part of the cellular activity—the nucleolus is especially prominent. Therefore an inquiry into its role in cellular metabolism is an appropriate topic for this Symposium.

Some years ago Dr. Mary Esther Gaulden and I (1) asked whether in a rapidly dividing cell, the grasshopper neuroblast, cell division would occur

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² Research supported in part by grant 12491 from the National Science Foundation.

if the nucleolus is damaged by selective irradiation with an ultraviolet microbeam. Except for nucleoli in late stages of the cell cycle, the answer was no. This suggested that nucleolar function is a prerequisite for cell division or for growth, since in this type of cell, growth is an obligatory concomitant of cell division.

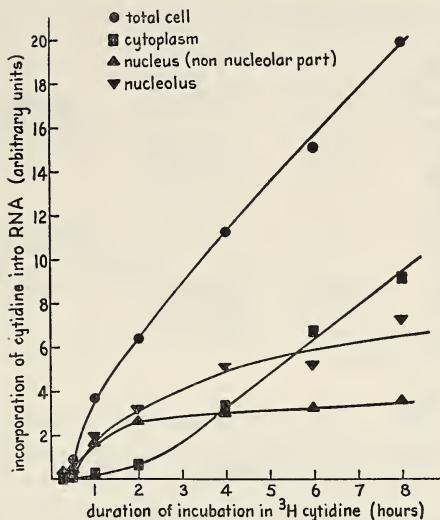
Because we desired to understand the nature of this control in molecular terms, Dr. Maurice Errera and I investigated the role of the nucleolus in RNA and protein synthesis. Our results (2-5), together with more recent work (6, 7), have led to the conclusion that nucleoli, or more correctly the nucleolar elements of the genome, direct the synthesis of ribosomal RNA. In terms of modern molecular biology this means that the nucleolus is involved with the production of the machinery for protein synthesis. Since each member of a dividing pair of cells must be endowed with essentially the same complement of protein-synthetic machinery as the parent, the production of ribosomes is a necessary property of a proliferating cell system. Hence the key role of the nucleolus in such systems becomes readily understandable.

In this communication I shall outline some evidence which supports these contentions: 1) experiments on the cytological level, designed to tell us something about the interrelationship between RNA's of the nucleolus, extranucleolar portions of the nucleus, and cytoplasm; 2) attempts to identify these RNA's with the biochemical terms: ribosomal, messenger, and transfer RNA; and 3) efforts to understand the chemical mechanism of the formation of ribosomal RNA.

KINETICS OF INCORPORATION OF NUCLEOSIDE PRECURSORS INTO RNA

With autoradiography one can study the synthesis of RNA at various sites within the cell. When rapidly growing tissue culture cells are incubated with labeled precursors, such as tritiated nucleosides, one observes a distinctive pattern of incorporation at the cellular level. If the cells are incubated for a relatively short period with radioactive precursor (pulse), one finds that incorporation occurs exclusively in the nucleolus and the extranucleolar (chromatin) portion of the nucleus. If the period of incubation is extended, or if the pulse is followed by a longer incubation with unlabeled precursor (chase), then one observes an appreciable amount of labeled RNA in the cytoplasm.

Quantitative measurements of the incorporation into the RNA of tissue culture cells may be obtained if one is careful to account for the uneven cell geometry (5). Text-figure 1 illustrates a set of kinetic curves for the incorporation of H^3 -cytidine into HeLa cells. The curves for nucleolar and chromatin incorporation are characterized by a rapid rise and early saturation, whereas the cytoplasmic incorporation shows an appreciable lag and no evidence of saturation for intervals as long as one generation



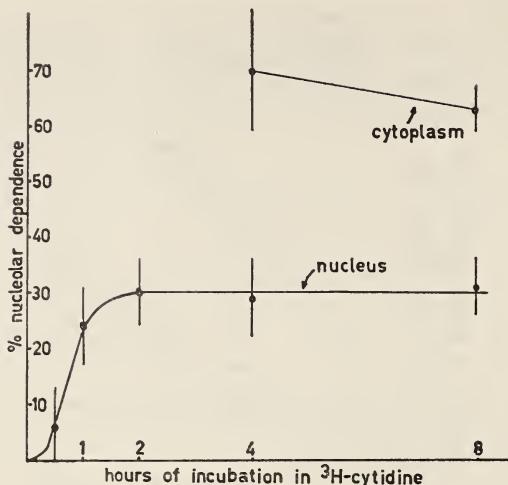
TEXT-FIGURE 1.—Incorporation of ^{3}H -cytidine into RNA of various parts of HeLa cells as a function of time. The ordinate is obtained from grain counts corrected for tissue self-absorption (5).

time. In contrast, the incorporation kinetics of amino acids into protein show no lag and are basically similar in all three compartments (4).

The kinetics of nucleoside incorporation are consistent with the idea that nucleolar RNA and/or chromatin RNA are precursor(s) of cytoplasmic RNA. However, another interpretation that postulates different and separately maintained pools of acid-soluble intermediates in the nucleus and cytoplasm could also be advanced. To obtain a more definitive answer to this problem we turned to microbeam irradiation and experiments with chemical inhibitors.

IRRADIATION OF NUCLEOLI WITH THE ULTRAVIOLET MICROBEAM

Irradiation of a HeLa cell nucleolus for a few seconds with an intense ultraviolet microbeam 2μ in diameter abolishes practically all subsequent incorporation of precursor into nucleolar RNA (2). Under such conditions one may measure the levels of incorporation in other parts in the cell. From the difference between these levels and those observed in the presence of normally functioning nucleoli, one can determine the "nucleolar dependence" of the RNA's in the other cell parts (3). Text-figure 2 illustrates the nucleolar dependence for the cytoplasm and the extranucleolar parts of the nucleus measured for various periods of incubation after irradiation of the nucleolus. Incorporation of label into the cytoplasm of irradiated cells is only 30 percent of normal: It may thus be said to be 70 percent nucleolar-dependent. Nuclear activity is roughly



TEXT-FIGURE 2.—Percentage of cytidine incorporation, in the cytoplasm and in the chromatin portion of the nucleus of HeLa cells, which is dependent on normally functioning nucleoli. The ordinate, nucleolar dependence, is defined as the difference in incorporation between control cells and nucleolar-irradiated cells, expressed as a percentage of the incorporation in control cells. The abscissa is the duration of incubation in ^3H -cytidine after nucleolar irradiation (3).

70 percent of normal, and thus 30 percent nucleolar-dependent. For very short incubation times the incorporation into nuclear RNA has essentially no dependence on the nucleoli. Hence there is good evidence for a strong nucleolar influence on the production of cytoplasmic RNA and for the relative independence of nucleolar and chromatin RNA syntheses.

Protein synthesis is not directly dependent on the nucleolus. In cells with irradiated nucleoli neither the cytoplasm nor the extranucleolar portions of the nucleus show any decrease in amino acid incorporation until more than half a cell generation has elapsed (4). Thus there seems to be only a remote dependence of protein synthesis on the nucleolus.

EFFECTS OF ANALOGUES AND INHIBITORS

It was found that certain chemical agents could also be used to induce specific changes in the intracellular pattern of RNA synthesis. In particular, the guanine analogues, 8-azaguanine and 2,6-diaminopurine, and the inhibitor, actinomycin D, are quite effective for this purpose. These compounds are of particular value because much is known concerning their chemical behavior and involvement with nucleic acid metabolism. Furthermore, by using them judiciously, one can prepare large quantities of cells in which the distribution of newly synthesized RNA is altered in a well-defined way. From such cells it is possible to extract and analyze RNA's which may be identified with particular cell sites.

TABLE 1.—Effect of azaguanine on the intracellular distribution of label in strain L fibroblasts (grains/30 cells, not corrected for self-absorption)

Labeling conditions	Cell part	Control	Azaguanine-treated*	Azaguanine control
30 minute pulse, H^3 -cytidine	Nucleolus	628	661	1.0
	Chromatin	591	698	1.2
	Cytoplasm	—	—	—
30 minute pulse, H^3 -cytidine, 4 hour chase, excess unlabeled cytidine	Nucleolus	824	779	0.9
	Chromatin	1,072	896	0.8
	Cytoplasm	3,757	882	0.2

*Azaguanine added to a final concentration of 2.5×10^{-4} M, 2.5 hours before pulse labeling.

Cells incubated with 8-azaguanine support the normal amount of RNA synthesis in both the nucleolar and chromatin portions of the nucleus. However, the appearance of newly formed RNA in the cytoplasm is greatly inhibited (8). Results from an autoradiographic experiment on strain L fibroblasts are shown in table 1. One observes the most marked effects when the cells are incubated with azaguanine for a few hours preceding the incubation with the RNA precursor. It could be further demonstrated that the cells incorporate C^{14} -8-azaguanine into nucleolar and chromatin RNA but not into DNA. Very similar effects are produced by 2,6-diaminopurine, whereas amino acid analogues have no apparent effect on RNA synthesis (9).

Actinomycin D at concentrations greater than or equal to 10^{-6} M inhibits essentially all RNA synthesis in mammalian cells (10). There is considerable evidence indicating that this inhibitor acts by binding to DNA and preventing its function as a template in the RNA polymerase system (11, 12). Yet, at concentrations between 10^{-7} and 10^{-8} M, there is appreciable RNA synthesis occurring almost exclusively in the chromatin portion of the nucleus. At these low concentrations of actinomycin, the synthesis of nucleolar RNA and the appearance of newly synthesized RNA in the cytoplasm are drastically suppressed (7). Figure 1 and table 2 illustrate how actinomycin at 3 to 4×10^{-8} M affects the incorporation of

TABLE 2.—Effect of actinomycin on the intracellular distribution of label in strain L fibroblasts (grains/30 cells, not corrected for self-absorption)

Labeling conditions	Cell part	Control	Actinomycin treated*	Actinomycin control
30 minute pulse, H^3 -cytidine	Nucleolus	984	384	0.4
	Chromatin	757	689	0.9
	Cytoplasm	95	—	—
30 minute pulse, H^3 -cytidine, 4 hour chase, excess unlabeled cytidine	Nucleolus	660	218	0.3
	Chromatin	906	1,163	1.3
	Cytoplasm	3,211	747	0.2

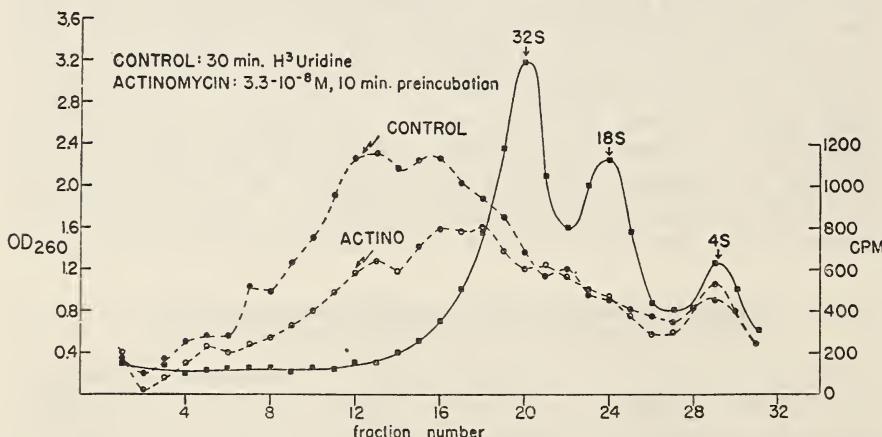
*Actinomycin added to a final concentration of 3.3×10^{-8} M, 30 minutes before pulse labeling.

H^3 -uridine and H^3 -cytidine. Here, as in the microbeam irradiation experiments, we have evidence of a relationship between nucleolar and cytoplasmic RNA and of the relative independence of chromatin RNA. This is in harmony with the work of Edström and co-workers who demonstrated that nucleolar RNA, but not chromatin RNA, has an average base composition similar to that of cytoplasmic RNA (13, 14).

SEDIMENTATION ANALYSIS

Rapidly Labeled RNA

Extraction with phenol and sodium dodecyl sulfate at 60° C allows virtually all of the RNA including the rapidly labeled fractions to be obtained in an undegraded form. When this RNA is layered on top of a linear sucrose gradient and centrifuged at high speed for an appropriate time, it is resolved into several distinct fractions (text-fig. 3). The stable RNA, shown by the solid line, consists of two components characteristic of ribosomal RNA (32 and 18S) and a 4S component which represents transfer RNA. The rapidly labeled RNA, that is, the nucleolar and chromatin RNA which is labeled during a 30-minute pulse of nucleoside precursor, is much more polydisperse than the stable RNA. It consists of fast components in the range 38 to 45S, a distinct 4S component, and lesser amounts of RNA's with intermediate sedimentation rates (6, 15, 16). Rapidly labeled RNA from cells treated with a low concentration



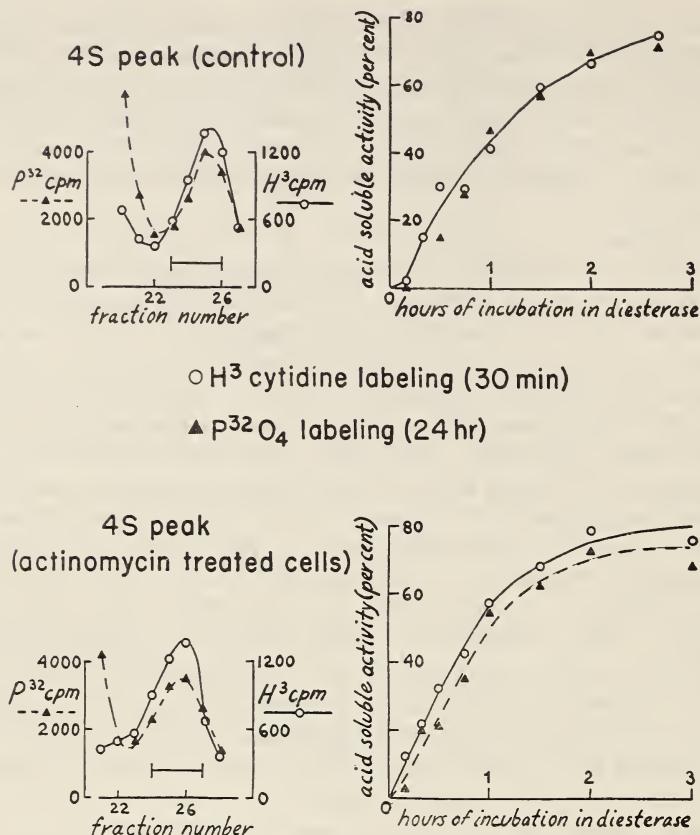
TEXT-FIGURE 3.—Effect of actinomycin on sedimentation pattern of rapidly labeled RNA from strain L fibroblasts. Extraction with phenol and sodium dodecyl sulfate at 60° C. Sucrose gradient 10 to 40 percent, SW 25,000 rpm, 10 hours. Faster moving components are at left. Solid curve represents the 260 m μ absorbance of the steady state RNA components. Dashed curves represent the cpm radioactivity in RNA from cells labeled for 30 minutes with H^3 -uridine. Note the diminution in heavier components observed in RNA from cells pretreated for 10 minutes with actinomycin.

of actinomycin, that is, RNA which is located almost exclusively in the chromatin region of the nucleus, contains a normal amount of 4S and intermediate components but is markedly deficient in the fast components (6, 17). From these data it may be concluded that the heaviest components of the rapidly labeled RNA are synthesized in the nucleolar region of the nucleus, whereas the lighter components and, in particular, the 4S component, are made in the chromatin region.

It is necessary to establish whether the incorporation of nucleoside which occurs in the presence of low concentrations of actinomycin represents true polynucleotide synthesis or whether it is just a terminal addition of nucleotide units to the ends of pre-existing chains. This is especially important in the case of the actinomycin-resistant 4S fraction because a turnover of the terminal nucleotides has been described for this component (18). To resolve this question we used the exonuclease property of snake venom diesterase. Cells were incubated with $P^{32}O_4$ for one generation so as to obtain a fairly uniform internucleotide labeling of the RNA, and then pulsed for 30 minutes with H^3 -cytidine. A portion of the cells were incubated with actinomycin shortly before and during the cytidine pulse. The RNA was extracted and resolved on sucrose gradients. The RNA in the 4S peaks was doubly labeled with H^3 and P^{32} (text-fig. 4). The 4S RNA's were isolated from the gradients and incubated with the snake venom diesterase. The production of acid-soluble H^3 and P^{32} activity was followed as a function of time. If a significant part of the cytidine incorporation were terminal addition, then one could expect a much more rapid increase of H^3 activity than of P^{32} activity, since the latter represents a degradation of a known internucleotide label. However, as is seen in text-figure 4, there is no difference in the kinetics of control RNA and only a slight difference with RNA from actinomycin-treated cells. Thus it is concluded that the incorporation of nucleoside in the chromatin region of the nucleus which is observed in the presence of low concentration of actinomycin D represents the synthesis of entire polynucleotide chains.

Ribosomal RNA

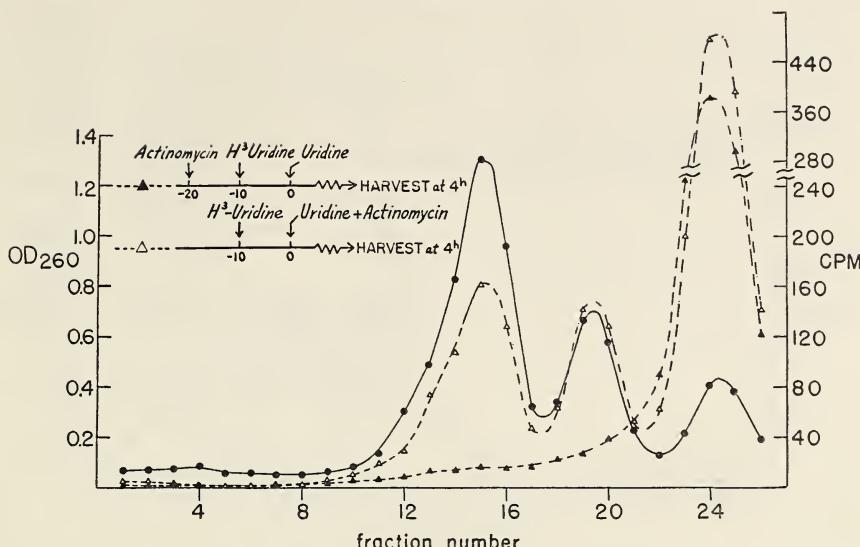
When a pulse with labeled nucleoside is followed by a 4-hour chase incubation, roughly two thirds of the labeled RNA is in the cytoplasm. Sedimentation analysis of RNA from such cells shows the bulk of the labeled RNA to be distributed in the three peaks characteristic of stable RNA. Thus, concomitant with a change in location of label from exclusively nuclear to predominantly cytoplasmic, there is a change in size distribution from one which is polydisperse to one consisting of three discrete components. In cells in which nucleolar RNA synthesis is blocked with actinomycin, there is no formation of ribosomal RNA. During this time there is a complete turnover of the intermediate-sized RNA's and an accumulation of the 4S component (text-fig. 5, solid



TEXT-FIGURE 4.—*Left:* 4S portions of sedimentation diagrams of RNA from L cells labeled for 24 hours with $P^{32}O_4$ and pulsed for 30 minutes with H^3 -cytidine. Extraction and gradient conditions as in text-figure 3. *Top*—RNA from control cells; *bottom*—RNA from cells treated for 10 minutes with $3.3 \times 10^{-8} M$ actinomycin D. Solid line indicates the H^3 radioactivity, dashed line the P^{32} activity. Horizontal bars indicate the fractions selected for degradation experiment. *Right:* kinetics of degradation of RNA isolated from the corresponding 4S peaks. The fraction of the total P^{32} and H^3 activity that appears in the acid-soluble supernatant is plotted against time of incubation with snake venom diesterase. Conditions of incubation with enzyme: 50 μ g RNA, 300 μ g purified venom phosphodiesterase at 37° C in 2.5 ml of 0.05 M glycine buffer, pH 8.4, containing 0.005 M $MgCl_2$.

triangles). If one allows the normal amount of pulse-labeled RNA to be formed and then subsequently chases the label in the presence of actinomycin, there is a substantial formation of ribosomal RNA (text-fig. 5, open triangles).

In the two experiments shown in text-figure 5, the total time of exposure to actinomycin is in one case 4 hours 20 minutes, and in the other, 4 hours. Since the 20-minute difference is only about one third the time necessary to produce ribosomal RNA (see text-fig. 6), it may be concluded that the critical difference between the two experiments is not the duration of

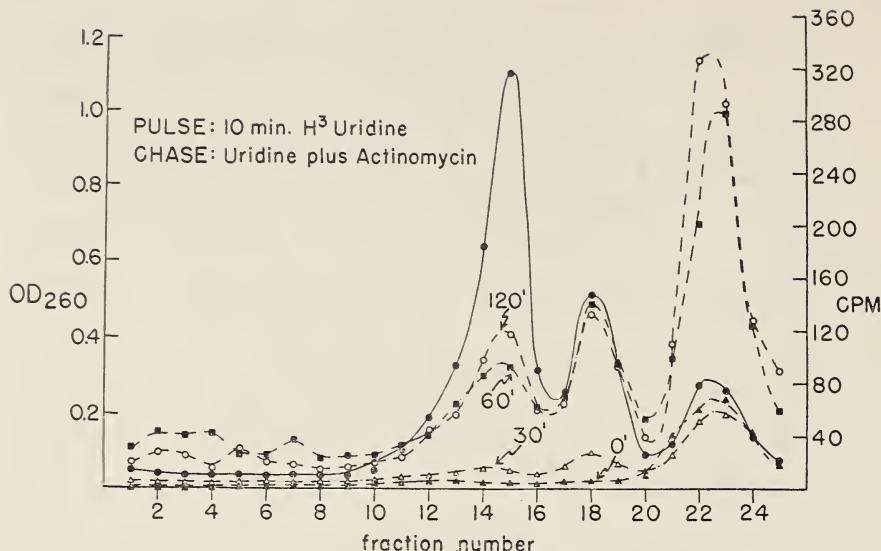


TEXT-FIGURE 5.—Effect of actinomycin on the formation of ribosomal RNA in L cells. Extraction with phenol at 23° C. Sucrose gradient 10 to 40 percent, SW 39,000 rpm, 3 hours. Solid curve as in text-figure 3. Dashed curves are cpm in RNA from cells labeled for 10 minutes with H^3 -uridine and chased for 4 hours with excess unlabeled uridine. ▲: 3.3×10^{-8} m actinomycin D added to medium 10 minutes before pulse with H^3 -uridine. △: 3.3×10^{-8} m actinomycin added to medium immediately after pulse with H^3 -uridine.

exposure to actinomycin, but rather that in one case the actinomycin is added before the pulse of labeled nucleoside and in the other it is added after the pulse. Hence, a blocking of the synthesis of 45S nucleolar RNA results in a blocking of ribosomal RNA synthesis. If the nucleolar RNA is produced, then it is transformed into ribosomal RNA *in the presence of the blocking agent*. These experiments clearly demonstrate that the 45S nucleolar RNA is an obligatory precursor of ribosomal RNA. Similar conclusions have been arrived at by other workers (17, 19).

A disproportionately large amount of 4S RNA accumulates under these conditions because the low concentration of actinomycin does not block its continued synthesis from the pools of labeled intermediates which are formed during the brief pulse. These pools do not contribute to ribosomal RNA because the production of nucleolar RNA is blocked by the actinomycin within a few minutes after the beginning of the chase period.

The abrupt "turning off" of the incorporation of labeled intermediates which occurs when chase incubations are performed in the presence of actinomycin may be used to determine precisely the kinetics of the formation of ribosomal RNA from a limited amount of precursor. In this study, one must prevent the large amount of rapidly synthesized nuclear RNA from obscuring the newly formed ribosomal components. This may be accomplished by use of a phenol extraction procedure yielding only ribosomal and 4S RNA (6, 15). The time sequence of the formation of



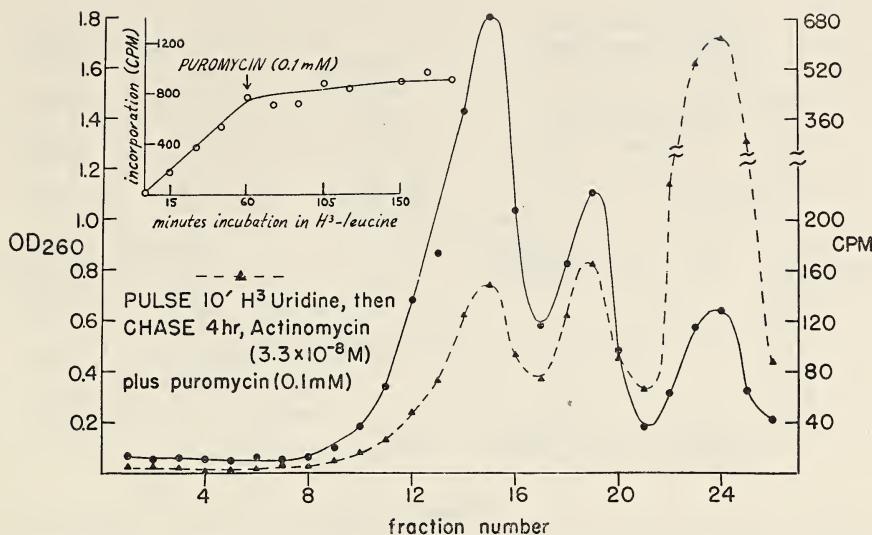
TEXT-FIGURE 6.—Kinetics of the formation of ribosomal RNA in L cells. Extraction and gradient conditions as in text-figure 5. Solid curve as in text-figure 3. Dashed curves are cpm in RNA from cells labeled with H^3 -uridine for 10 minutes and chased with excess unlabeled uridine in the presence of 3.3×10^{-8} M actinomycin D. Numerals over the curves indicate the duration of chase in minutes.

ribosomal RNA in text-figure 6 shows that 60 to 120 minutes are necessary for the complete transformation of 45S precursor to 18 and 32S units.

TRANSFORMATION OF NUCLEOLAR RNA INTO RIBOSOMAL RNA

It was surprising to find that newly formed nucleolar RNA has a sedimentation constant of 45S, which is considerably larger than either of the ribosomal RNA units. Yet the average base composition of this precursor RNA closely resembles that of the ribosomal RNA (17). One is therefore led to search for some important—but as yet unknown—steps intervening between the synthesis of nucleolar RNA and its transformation into a stable ribosomal RNA. From the experiments employing azaguanine, it would appear that this transformation requires the precursor to contain the proper bases rather than their close relatives. Although there is normal formation of an azaguanine-containing precursor, there is very little formation of ribosomal RNA. A complication of these experiments, however, is that azaguanine also suppresses protein synthesis (8), and one might argue that it is this aspect of its action which blocks the transformation. Therefore, we investigated the action of another potent inhibitor of protein synthesis, puromycin.

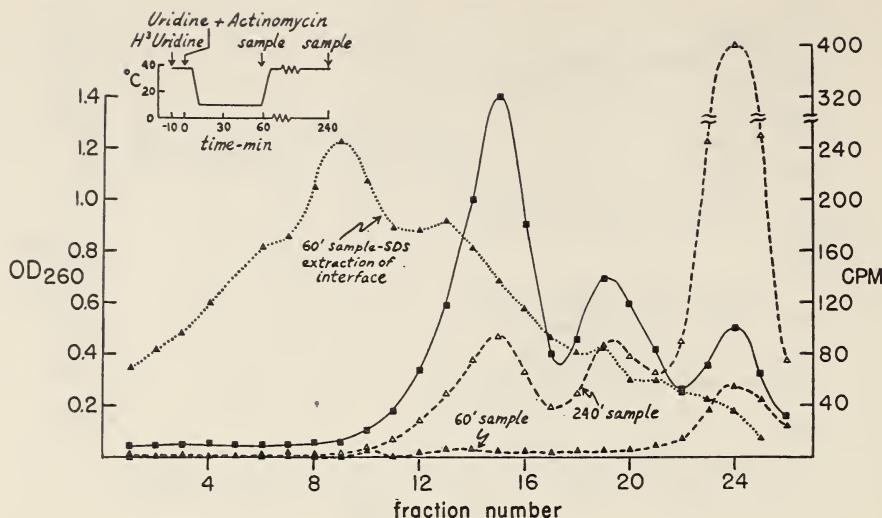
At 10^{-4} M puromycin produces a very rapid and almost complete inhibition of protein synthesis (see inset, text-fig. 7). If one gives cells a



TEXT-FIGURE 7.—Effect of puromycin on the transformation of precursor into ribosomal RNA in L cells. Extraction and gradient conditions as in text-figure 5. Solid curve as in text-figure 3. Dashed curve is cpm in RNA from cells labeled with H^3 -uridine for 10 minutes and chased for 4 hours with excess unlabeled uridine in the presence of 3.3×10^{-8} M actinomycin D and 10^{-4} M puromycin. Inset illustrates a separate experiment which was performed to show the abrupt cessation of protein synthesis caused by the addition of puromycin at a concentration of 10^{-4} M. In this experiment the incorporation of H^3 -leucine into acid-precipitable protein was used as a measure of protein synthesis. It is seen that the rate of incorporation drops practically to zero immediately after the addition of the puromycin.

short pulse of H^3 -uridine and then further incubates in a chase medium containing actinomycin and puromycin, one finds that an amount of ribosomal RNA is produced which is essentially the same as that made in the presence of actinomycin alone (cf text-figs. 5 and 7). Thus, if the transformation depends on protein, it is not a newly synthesized protein.

As a basis for future work we investigated the temperature sensitivity of this transformation. The experiment is shown in text-figure 8. Cells were given a 10-minute pulse of H^3 -uridine. Unlabeled uridine and actinomycin were added to the medium, and the temperature was rapidly lowered from 37° to 5° C. After 1 hour a sample of cells was taken for analysis. The remaining cells were warmed to 37° C, kept there for 3 hours, and then also harvested for analysis. Clearly, although 60 minutes at 37° C is sufficient for the transformation into ribosomal RNA (cf text-fig. 6), no ribosomal RNA is made during an equivalent period at 5° C (text-fig. 8, closed triangles). An analysis of the RNA in the water-phenol interphase showed that in the chilled cells the RNA retained the form of the rapidly synthesized precursor, even after 60 minutes. When the cells are warmed to 37° C, they can again effect the transformation, although there appears to be some lag in regaining this ability (text-fig. 8, open triangles).

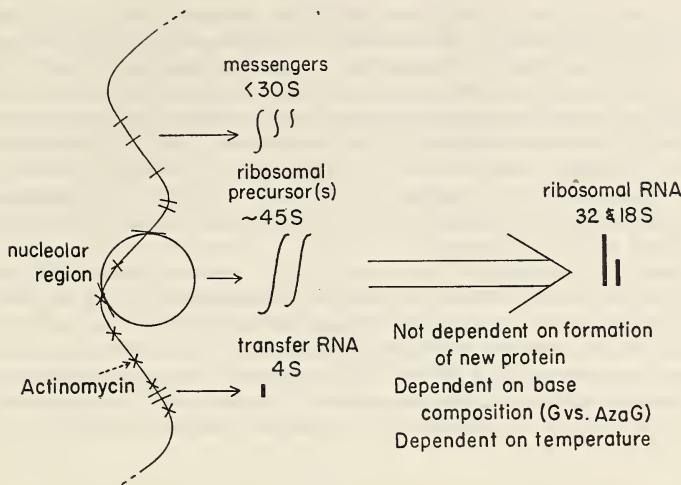


TEXT-FIGURE 8.—Temperature dependence of the formation of ribosomal RNA in L cells. Gradient conditions as in text-figure 5. The 60-minute sample was first extracted with phenol, 23° C (dashed curve) and then the interphase was further extracted with phenol and sodium dodecyl sulfate, 60° C (dotted curve). The 240-minute sample was extracted with phenol, 23° C. Inset shows the time-temperature relationships and the times of additions and withdrawals of samples.

ROLE OF THE NUCLEOLUS IN RNA SYNTHESIS

From the foregoing data we are led to propose the following scheme for RNA synthesis in the intact cell (text-fig. 9). The DNA of the genome, shown schematically as a linear thread, dictates the synthesis of various types of RNA. The nucleolar regions are concerned with the production of ribosomal precursors in the form of large units, roughly 45S. The other regions of the genome, that is, those in the chromatin portion of the nucleus, are involved in the production of transfer (4S) RNA's and of RNA's of intermediate sizes. The latter might be the messengers which are used to code for the synthesis of specific proteins. The production of ribosomal RNA by a few discrete nucleolar regions has an analogy in the case of bacteria where the cistrons for ribosomal RNA are also thought to be confined to a discrete portion of the chromosome (20, 21). That there should be a relatively short portion of the total genome endowed with the information for ribosomal RNA production is understandable, since organisms of widely varying DNA base composition have ribosomal RNA's which are quite similar in composition (22).

The fact that at low concentrations actinomycin D has a preferential inhibitory action on nucleolar RNA synthesis may have a rather simple explanation. It is known that actinomycin binds specifically to guanine residues of DNA (11), and one may envision that an RNA molecule which is being copied sequentially from a DNA strand would have its synthesis



TEXT-FIGURE 9.—Schematic diagram of RNA synthesis in the intact cell. For explanation, see text.

terminated when it reached a guanine base that was bound to actinomycin. This would result in either a shorter than normal RNA strand or perhaps even a completely unstable entity. At low concentrations the guanines that are bound would be rather widely separated. Under these conditions it might be possible that the smaller messengers and especially the 4S RNA are read out between the binding sites. On the other hand, the nucleolar RNA, being the longest piece, would have the highest probability of being terminated before reaching its normal length and hence would be most sensitive at the lowest concentrations. This probability would be further augmented because the nucleolar RNA has a high C-G base composition which would predict that its complementary DNA would have more binding sites for actinomycin.

The mechanism by which the nucleolar RNA is transformed into the two ribosomal components is still unclear. Although it does not depend on the synthesis of new protein it does seem to involve a temperature-dependent reaction that requires the precursor to contain the proper bases. Since ribosomal RNA is thought to have a considerable amount of secondary structure comprising double-stranded segments (23), whereas the precursor is most likely a single-stranded entity, this transformation may involve some type of folding and base-pairing reaction.

RESUMEN

Se ha mostrado que la función nucleolar es indispensable en las células que están continuamente creciendo y dividiéndose. Con más propiedad ésto se relaciona con el hecho de que el núcleo es un lugar especial para la producción de ácido ribonucléico (RNA). Las siguientes líneas de evidencia se presentan para demostrar que el RNA

nucleolar es un precursor obligatorio del RNA ribosomal del citoplasma: (i) La dinámica de la incorporación de nucleótidos marcados radiactivamente en las fracciones del RNA nucleolar y citoplásmico concuerdan con una relación precursor-producto. (ii) La supresión de la síntesis de RNA nucleolar por irradiación selectiva de los nucleolos con una microemisión ultravioleta conduce a una marcada reducción ($\sim 70\%$) en la nueva formación de RNA citoplásmico. (iii) El tratamiento de las células con una baja concentración de actinomicina D bloquéa selectivamente la síntesis de RNA nucleolar y conduce a la completa inhibición de la síntesis de RNA ribosomal; sin embargo el tratamiento con actinomicina no inhibe la conversión de RNA nucleolar a RNA ribosomal.

En análisis de sedimentación indica que el RNA nucleolar nuevamente sintetizado tiene una sedimentación constante de 45S aproximadamente lo que es considerablemente más grande que cualquiera de los componentes del RNA ribosomal (18 y 32S). Este hecho nos ha conducido a una exploración de los mecanismos que emplean las células para transformar RNA 45S en RNA ribosomal. La transformación no ocurre a $5^\circ C$ y aparentemente es inhibida cuando el precursor RNA contiene azaguanina. Por otra parte, no parece depender de la formación concomitante de nueva proteína.

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PLATE 8

FIGURE 1.—Autoradiographs of HeLa cells that were pulse-labeled for 5 minutes with H^3 -uridine and then incubated for 30 minutes in medium with excess unlabeled uridine: (a) control; (b) incubation with 4×10^{-8} m actinomycin D for 30 minutes immediately preceding, and 5 minutes during, the pulse with H^3 -uridine. Note that although there is a marked difference between the intensity of labeling in the nucleoli, there is relatively little difference in the amount of label in the chromatin portion of the nucleus (?).



Cellular Alterations by Viruses and the Induction of Cancer

Functional Alterations of Cells Due to Viruses^{1, 2}

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SUMMARY

Viruses can produce permanent cellular alterations in two ways: either by initiating new synthetic processes directed by viral genes or by establishing new regulatory controls on the function of cellular genes. Examples of the first type of mechanism are presented, derived from the study of both bacterial and animal viruses. Possible examples of the second type of mechanism are also discussed. It is shown that

the manifestations of virus-directed function are often dependent on the genotype and the physiological and developmental state of the host cells. The role of virus-induced cellular alterations in the determination of the response of cells to environmental factors is examined. Implications of these phenomena for the problem of carcinogenesis are presented.—Nat Cancer Inst Monogr 14: 93-106, 1964.

THE PROBLEM discussed here concerns the mechanisms by which viruses alter their host cells. The alterations that especially concern us in the framework of this Symposium are those persisting through a cell lineage, as observed in tumors. Any discussion of persistent cellular changes elicited by viruses necessarily revolves around the current concept of virus infection as genetic infection or infective heredity (1-3).

A virus is not a cellular, but a subcellular entity. It exists in two mutually exclusive states: the virus particle, or virion, and the viral genome. The virion is a terminal product of virus maturation. It protects the viral genome within and outside the host cell and facilitates its entry into other cells. The few specific functional activities exerted by components of the virion other than the viral genome—attachment and penetration functions in some phages, neuroaminidase activity in myxoviruses, and intracellular release of the viral genome in poxviruses (4)—are, indirectly, activities of the viral genome since they are exerted by virus components synthesized under the control of the viral genes. In addition, these functions of virions are characteristically transitory ones. As such, they

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² This paper is based on the R. E. Dyer Lecture presented at the National Institutes of Health, Bethesda, Md April 24, 1963.

are unlikely to exert any permanent hereditary effects on their host cells, although it cannot *a priori* be excluded that a transient alteration, of the cell surface or of the enzymatic balance of a cell, may trigger lasting and permanent effects [see (5) for discussion]. The example of chemical carcinogens may be recalled in this connection.

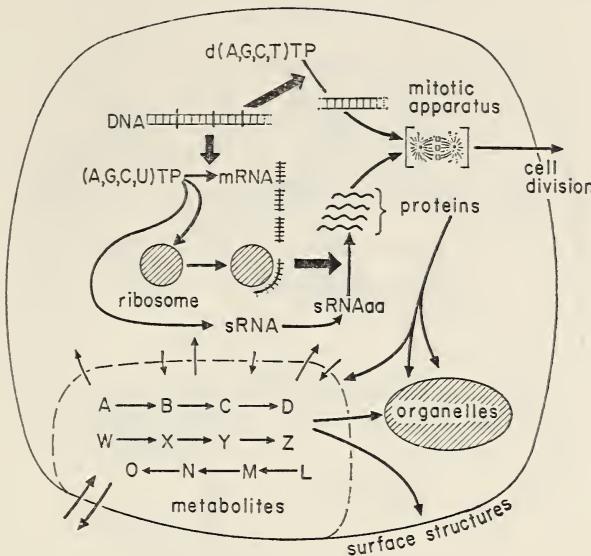
It seems altogether likely that most of the persistent cellular alterations that follow virus infection reflect the function of the viral genome, which should be considered as an accessory, more or less compatible adjunct to the cellular genome.

This concept of virus infection explains why virology is rapidly becoming a branch of genetics. Also, it brings the modern advances in molecular genetics to bear on the cellular pathway of viral diseases. It is reasonable to assume that the molecular mechanisms of gene action will be similar whether they are exerted by cellular or by viral genes. The informational content, hence the nature of the functions, will of course be different, as also may be the regulatory mechanisms that control gene action. In fact, any significant departure from the common pattern of gene action revealed by the study of viruses is bound to reveal some novel features of the genetic material and its activity. It is sufficient to mention here the problems of ribonucleic acid (RNA) genes and their regulation raised by the existence of RNA viruses.

In the present paper, four topics will be dealt with: 1) the place of viruses in the functional organization of cells; 2) the function of structural genes of viruses; 3) the regulatory functions affecting virus development; and 4) a novel class of alterations of genetic material revealed by the study of viruses. Most of the discussion will use examples from the field of bacterial viruses, with some examples from tumor viruses, to illustrate certain principles of viral physiology rather than to interpret the role of viruses in the etiology of tumors. It should be stated clearly that the thesis presented in this paper is not that all tumors reflect the action of viruses, nor that all tumors stem from changes in the amount or structure of the genetic information of cells; rather, it is proposed that any effects of viruses on cells, including those that can lead to tumor cell formation, may be traceable, directly or indirectly, to specific activities of the viral genome.

VIRUSES AND CELLULAR ORGANIZATION

Text-figure 1 is a schematic representation of cellular organization illustrating the functional hierarchy of cellular components according to current theories. The deoxyribonucleic acid (DNA) of the genes is shown as the source of genetic specificity, directing its own replication and, through RNA messages, the synthesis of proteins. These in turn exert the catalytic functions and together with derived macromolecular species provide the mechanical framework of cellular structure. DNA as well as proteins and their products are functionally coordinated in the events lead-



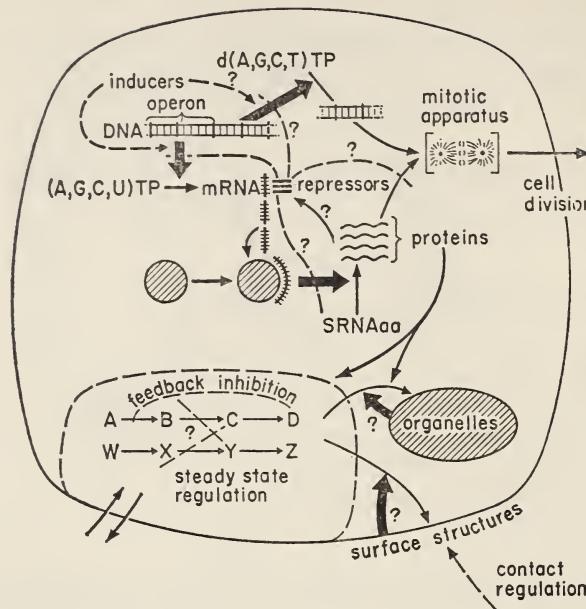
TEXT-FIGURE 1.—Diagrammatic representation of the functional organization of cells.

Heavy arrows indicate template functions; light arrows indicate precursor-product relations. mRNA and sRNA stand for messenger RNA and soluble RNA, respectively.

ing to cell division, represented by mitosis in all but the prokaryotic organisms, *i.e.*, bacteria and their relatives (6).

Besides the structural genetic information for specific cellular components, a cell requires regulatory mechanisms. Most of the time a majority of the genetic functions of a cell fail to be expressed. There are various levels of regulation (see text-fig. 2). Those exerted directly by specific regulatory genes (7) are of special interest for the present discussion. At least in bacteria, where they are best understood, the regulatory genes act by producing repressor substances which, operating directly at the gene level, control and regulate the activity of genes or groups of genes—the units of coordinated functions or operons. The repressors are activated or antagonized by specific substances of low molecular weight—co-repressors and inducers—either coming from outside the cell or of internal, metabolic origin. Repression is the main known mechanism through which the chemical milieu exerts its control over gene function in bacteria. Some of the humoral controls in multicellular organisms probably act in a similar way. Complex and refined regulatory systems may be constructed by interaction of multiple repression mechanisms, in parallel or in series (8, 9). The cycle of DNA replication and cell division is probably regulated by specific control mechanisms, whose study is still in its infancy.

Where do viruses belong in this scheme of cellular organization? Text-figure 3 illustrates some of the presumed relations. For the DNA viruses, the viral genome is assumed to function as cellular DNA does. Hence, it

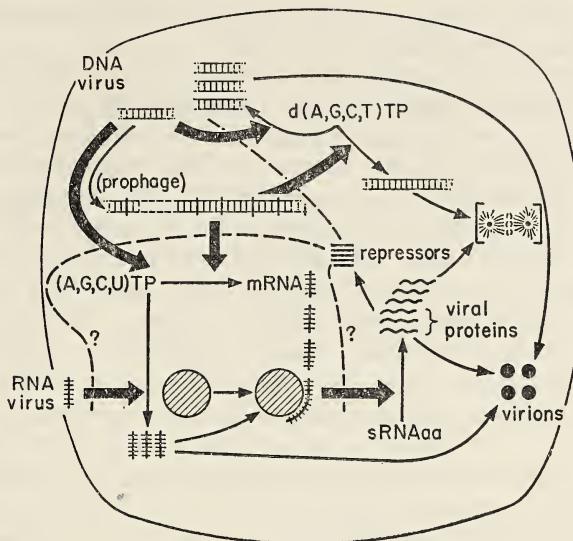


TEXT-FIGURE 2.—Regulatory mechanisms in cellular organization. *Broken lines* indicate regulatory effects of repressors or inducers. Mechanisms and relations whose existence is uncertain are indicated by *question marks*. The cell in the diagram is represented as from an eukaryotic organism (presence of a mitotic apparatus), even though most of the regulatory mechanisms shown are known only in bacteria.

presumably acts both as a template for replication and as a source of structural and regulatory information. The viral DNA may replicate separately and out of coordination with the cellular DNA. Or, in certain bacteriophages, the phage genome may become physically integrated as *prophage* within the cellular DNA. These phages are called temperate and can establish a fairly permanent lysogenic condition. This kind of physical integration with the host-cell genome has not yet been demonstrated for any animal viruses containing DNA, even for those, such as rabbit papilloma, that can persist within a multiplying cell line for many generations and possibly indefinitely.

Physical integration with the viral genome need not be a requirement, however, for persistence of a viral genome. In bacteria, besides the phages, there are other accessory DNA elements that can persist more or less permanently by replicating autonomously and in physical separation from the cell genome. These elements—plasmids (10) or episomes (11)—include the sex factors, the bacteriocinogenic factors, and some others. It is conceivable that the genome of some viruses might persist in the same way; in fact, some phages probably do (12).

The viruses whose genome consists of RNA introduce a new element in the scheme of cellular organization, as outlined in text-figures 1, 2, and 3, because this scheme provides no place for RNA genes. Whether or not such genes occur in normal cells apart from virus infection is not known.



TEXT-FIGURE 3.—An idealized picture of the role of viruses in cellular organization.

As in text-figure 2, the cell diagrammed is that of eukaryotic organism, but some of the viral mechanisms (such as the prophage integrated into the cell genome) refer to bacteria. Viral DNA is shown as source of template action for replication and production of messenger RNA. Viral RNA is shown as messenger RNA as well as template for its own replication. Virions may contain DNA or RNA.

[The chloroplasts, the cellular organelles with the most valid claim to genetic continuity, contain some distinctive DNA (13).] In directing protein synthesis, viral RNA acts as messenger RNA (14). For replication, however, it apparently depends on DNA-independent RNA polymerases, enzymes that have been found both in bacteria infected with the RNA phages (15) and in human cells infected with poliovirus (16). No such enzymes have yet been found in uninfected cells. It is conceivable that an RNA-primed RNA polymerase, once made in virus-infected cells under the control of a persistent RNA virus (for example, Rous sarcoma virus), might bring about, if it is not completely specific, permanent cellular alterations by copying some of the RNA messages from cellular genes. This intriguing possibility remains to be explored.

STRUCTURAL GENES OF VIRUSES

In order to ascribe to a gene and to the nucleic acid element containing that gene the information for the structure of a given protein, one needs to demonstrate that a set of mutations occurring in that gene (or the corresponding nucleic acid) is correlated with a set of changes in the structure of the protein—more specifically, in its amino acid sequence. Quantitative changes, including the total absence of the protein as a result of a

mutation, do not suffice as a criterion, because such changes may reflect regulatory rather than structural functions.

By this criterion one recognizes in viruses the presence of structural genes for the proteins of their virions. For example, in tobacco mosaic virus, poliovirus, as well as many phages, viral mutations are correlated with specific alterations in the structure of virion proteins recognizable chemically, functionally, or serologically (17, 18). Likewise, the lytic enzymes produced in bacteria infected with phage are coded by phage genes. In phages that have been used for extensive genetic analysis, the genes corresponding to several virion proteins have been mapped [see (18) for discussion of recent work]. The significance of these findings for the problem of persistent cellular changes controlled by viruses is limited, because in most instances the virion proteins fail to be made in cells that are persistently infected, for example, in lysogenic bacteria. It was suggested that virion proteins of Rous sarcoma virus might play a role in tumorigenesis by altering the surface properties of persistently infected cells (19). But the recent discovery that this virus is defective and cannot by itself cause production of virions (20) seems to render this hypothesis inadequate.

More interesting in the present context is the appearance of new enzymes in virus-infected cells. Many of these enzymes are directed to the biosynthesis of viral nucleic acids. Examples are: the already mentioned DNA-independent RNA polymerases; the enzymes formed after infection of coliform bacteria with temperate phages [including the T-even phages that contain 5-hydroxymethylcytosine in their DNA (21)]; and the DNA-synthesizing enzymes found in the cytoplasm of cells infected with poxviruses (22).

Some of the enzymes made in bacteria after infection with T-even phages have been shown to be structurally determined by viral genes, whose mutations cause the production of altered forms of enzyme (23, 24). The corresponding evidence for the phage RNA polymerase is indirect (25), although probably valid. The same may be said of the enzymes elicited by poxvirus infection. There is no obvious reason why similar enzymes might not be present in cells infected with persistent viruses. (The arginase of virus-induced rabbit papillomas is discussed by S. Rogers, this Symposium.)

The addition of new enzymes to the metabolic machinery of a cell is evidently a powerful mechanism by which a persistent virus could alter cellular functions. Yet, the very presence of the structural gene for an enzyme does not assure that that enzyme will in fact be present, as discussed in the following section.

REGULATORY FUNCTIONS AND VIRUS INFECTION

Just as cellular genes are subject to as well as generators of regulatory controls, so also are viral genes. The most remarkable instance of virus control over viral functions is observed in lysogenic bacteria (26). A

repressive mechanism is established—the immunity repressor, the product of a prophage gene—which prevents the expression of most other phage genes (not all!), including those determining virion proteins and lytic enzymes. Hence, the presence of the prophage can often be recognized only indirectly when the repression fails and the viral genome replicates rapidly and produces complete virions. If a prophage, already subject to the immunity repressor, undergoes any mutation that prevents the synthesis of complete virions it becomes a defective prophage, whose presence is even more difficult to detect. These two mechanisms, immunity and defectiveness, although known only in phage, have long been popular models for interpreting viral latency in virus-induced tumors. In this connection, we recall the already mentioned recent discovery (20) that Rous sarcoma virus is a defective virus, which can only produce mature virions when the cells that carry it are superinfected with a related nondefective viral agent.

Coordinate repression of viral genes is not limited to lysogeny. Thus, early after infection with a T-even phage, only the enzymes needed for DNA synthesis are produced, not the virion proteins. After new DNA appears, the virion proteins begin to form and the "early enzymes" stop increasing (21, 27). A similar reciprocal exclusion between synthesis of viral enzymes and virion proteins is observed in cells infected with poxviruses (28). The exact nature of the controlling mechanisms is not yet known, but probably reflects the activity of repressors produced by viral genes.³

Another set of regulatory effects of viruses concerns host-specific biosyntheses. Characteristically, those bacteriophages that have been called temperate (as well as certain animal viruses) block the synthesis of cellular proteins by abruptly interrupting the production of RNA messages from the cellular genes (30). This remarkable control mechanism in itself would not be relevant to persistent infections; it is too sweeping to allow continued life of the cell. But its mechanism may help us interpret more subtle and significant effects of viruses on regulation of cellular functions, such as are observed in long-lasting infections. A very interesting instance is that of rabbit papilloma virus. Infection occurs in the cells of the basal layer of the epidermis. Due to infection, these cells become able to divide even when they are not in contact with the underlying derma, something normal cells cannot do. The infected cells form masses of actively growing, dividing, nonkeratinized cells of the basal layer type, which contain no virion protein (31). When keratinization occurs and cell division stops, the viral proteins and the mature virus begin to be formed. In this instance the virus appears to act as a tumor-producing agent by altering the morphogenetic response of the cells to external stimuli, fostering cell multiplication and delaying keratinization. The mechanism of this remarkable effect of the virus on morphogenesis

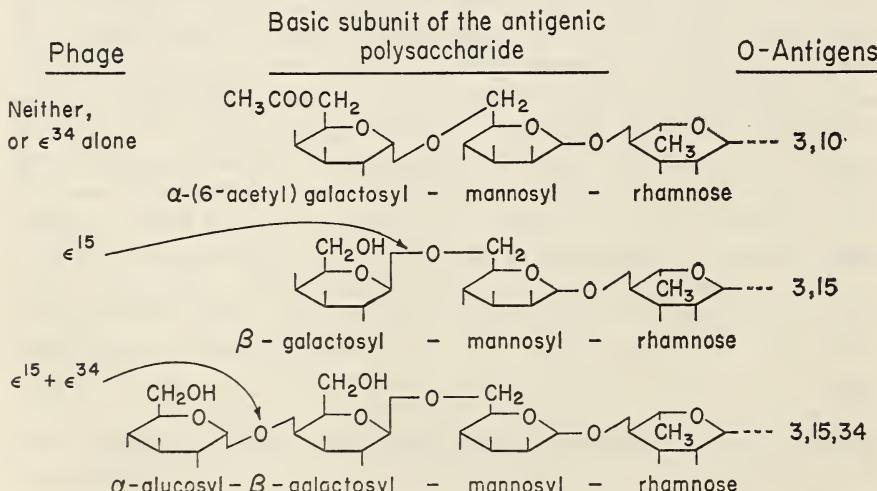
³ It is interesting that regulatory effects of the same kind have recently been noted with an RNA phage (29). This observation is especially important because it reveals that regulation and repression may be exerted not only at the level of DNA → RNA transcription, but also at the level of RNA → protein translation.

remains to be clarified. One may speculate that the reproductive capacity of the basal layer cells is maintained by a substance derived from the dermal layer, and that the virus-infected cells have a lower threshold of response to such substance—possibly because of enhanced permeation or active uptake.

CELL-CONVERSION PHENOMENA

The alteration of cellular properties of bacteria by phages has been called conversion. The same term should probably be applied to the changes in cellular properties and growth pattern produced by tumor viruses.

A typical example is found in bacteria of the genus *Salmonella*, many strains of which owe part of the antigenic specificity of their cell wall polysaccharides—the specific components of the O-antigens—to certain phages (32). In the group E *Salmonellas*, which has been analyzed in the greatest detail, the changes brought about by two phages have been traced to specific chemical changes (text-fig. 4). Infection with one phage, ϵ^{15} , causes the substitution of a β -linkage for an α -linkage in the side chains of the polysaccharide and a failure to acetylate a sugar residue. Infection with a second phage, ϵ^{34} , leads to addition of a glucose residue at a specific site on the side chains. These changes are extremely rapid. They appear a few minutes after infection, affect all newly synthesized polysaccharide molecules, and last as long as the viral genome is present. If the virus is lost, the polysaccharides return to being made in their original form. The actual mechanisms—production of new enzymes determined



TEXT-FIGURE 4.—The chemical nature of the side chains of the specific O-antigen polysaccharides of *Salmonella* E₁ (antigen 3,10), E₂ (antigen 3,15), and E₃ (antigen 3,15,34).

by phage genes, or repressions and derepressions of bacterial genes—are still under investigation.

Such a system has two interesting features. First, since the antigenic changes due to phage are only minor changes in the structure of the polysaccharides, they can be expressed only within the proper genotypic and phenotypic background. Thus, the converting activity of the virus is unrecognizable either in rough strains, which lack the O-antigens altogether, or under any physiological conditions in which the polysaccharides cannot be synthesized. This example illustrates the role of genetic and developmental properties of host cells in determining the nature of the cellular alterations that result from a virus infection.

Second, the conversions observed in *Salmonella* concern properties of the cellular surface. It is known that the surface of cells is the site of important, if poorly understood, regulatory controls—the so-called contact regulation. Surface changes evoked by viruses can alter the response of cells to their neighbors (see Pardee, this Symposium). The new antigens that have been found in cells converted by polyoma virus (33, 34) may have some relation to the malignant qualities of these cells.

HOST-INDUCED MODIFICATION: A NEW CLASS OF CHEMICAL CHANGES IN GENETIC MATERIAL

A group of phenomena, discovered first in bacteriophages and later also in other viruses, has revealed a totally unexpected type of interactions among genetic elements within a cell. These interactions may prove important in a variety of phenomena, including normal and abnormal differentiation, because they provide a method by which certain genes can specifically modify the structure of the DNA of other portions of genetic material, in a way that does not alter their coded information but interferes with their function and integrity.

The basic observations are as follows (35): Most bacteriophages can multiply in a series of related hosts. After reproducing in a certain host strain A, most particles of a given phage, X, often become unable to grow on another host, strain B, although they can still grow on A (host-range restriction). Those particles of phage X that succeed in multiplying in host B give a progeny which is again normal and capable of growing on either A or B (unrestricted phage). The same derestriction may follow one growth cycle of X on another host strain C. In some cases, the differences between bacterial strains A, B, and C are intrinsic properties of their genomes. In other cases, strain B may differ from A only in being lysogenic for some prophage, totally unrelated to phage X. The prophage sets up a rejection mechanism for the extraneous DNA of another phage. Similar effects have also been observed with a variety of animal viruses.

These observations on host-induced modification were neglected for several years because they seemed to concern some trivial role of host cells on the integrity of the virions produced within them. A completely

new light was thrown on them by the discovery (36, 37) that the host-induced restrictions are stable chemical changes in the nucleic acid of the virus; changes, however, which do not alter the genetic specificity, *i.e.*, the code sequence of bases in the nucleic acid, but only the ability of the nucleic acid to function and to be replicated. This was elegantly shown by Arber and Dussoix (37), who, using density labels, demonstrated that if phage X previously grown on host B multiplies for one cycle in host A, only the new particles whose DNA has been made in A are rejected by B, whereas the few particles that contain a strand of parental DNA (made in strain B) "remember" their previous host and are recognized and accepted by strain B. None of these changes are mutations: They do not alter any of the hereditary properties of the phages.

What is the nature of the chemical changes involved? In one case this has been clarified by work recently done in our laboratory (38). Phage T2 and its relatives contain in their DNA the peculiar base 5-hydroxymethylcytosine (HMC) instead of cytosine, and the HMC in their DNA becomes glucosylated by glucosyl transferases which use uridinediphosphoglucose (UDPG) as glucose donor [see review by Cohen (21)]. If these phages are grown in bacteria defective in the production of UDPG, the yield consists of particles with nonglucosylated DNA, which cannot multiply on most strains of *Escherichia coli*, but can still multiply in *Shigella dysenteriae*, yielding normal, glucosylated phage.

Most instances of host-induced restrictions must be due to some other mechanism, because HMC and its glucosylated form are not present (at least not in detectable amounts) in the nucleic acids of other viruses. The widespread nature of the phenomena suggests a more accessible mechanism. A favorite hypothesis is that host-induced modifications may be due to enzymatic, selective methylation of purine and pyrimidine bases at certain sites in DNA (and in RNA, for RNA viruses). Selective methylation, such as is known to occur (39, 40), may expose the viral nucleic acid to, or protect it from, inhibitory and destructive actions of cellular enzymes. In some cases the DNA of restricted phages is actually degraded to acid-soluble fragments in the bacteria that reject it (37, 41).

The remarkable aspect of these phenomena is that they provide selective mechanisms for the controlled rejection of extraneous nucleic acids. They are isolating mechanisms at the molecular level and are not limited to phage. The same bacteria B that reject the DNA of phage X previously grown on bacteria of strain A will also tend to reject the DNA of strain A itself if they receive it, *e.g.*, in the course of mating (41).

What implications do these findings have for the biology of cancer? Several possibilities suggest themselves. First, a virus or provirus in a cell may initiate alterations of the cellular DNA leading to inhibition or even destruction of certain parts of the cell genome, in the same way as a prophage can cause a bacterium to reject unrelated phages.

Second, acceptance or rejection of a virus, and the results of infection in a multicellular, differentiated organism may be controlled by the activity, in some tissues and not in others, of restricting gene function. Thus, a

tumor virus may function only in certain lines of cells that have acquired, or lost, specific functions in the course of development.

Third, interactions like those of modification and rejection of viruses by different cells may also occur among portions of the genome of the same cell. For example, the activation in the course of development of a gene, producing, say, a certain methylating enzyme, may lead to permanent inhibition or destruction of certain other genes. Permanent, quasi-irreversible patterns of differentiations might be established in this way. The reverse might equally occur, leading to reactivation of blocked cellular potentialities and, possibly, to abnormal developmental patterns as in cancer. In fact, this might be the mode of action of certain tumor viruses.

The significant aspect worth stressing is that these phenomena reveal a new level at which the function of genes is specified. Besides the genetic code and the regulatory mechanisms of the repressor-inducer type (7), one must now take into account also the phenomena exemplified by host-induced modification, in which a fragment of the genome determines the fate and function of entire classes of genetic materials.

CONCLUSIONS

The thesis of this paper is that viruses act on cells as added pieces of genetic material which control new syntheses, exert new regulatory functions, and are in turn regulated by the cellular milieu. Hence, any cellular abnormalities due to viruses, including abnormal patterns of growth and development, must reflect directly or indirectly the functions of the viral genome. According to this viewpoint, there is no reason to postulate that certain classes of persistent cellular abnormalities such as cancer should result only from the activity of viruses, just as there is no reason to believe that all genetic changes in bacterial polysaccharides are due to phages. Since viruses are represented in the host cells by elements of genetic material which are not fundamentally different from those of the cell, it is reasonable to suppose that what a virus can do to the growth regulation and differentiation of a cell, other classes of genetic changes can do as well. An alternative hypothesis would be that there is a more unique relation between viruses and cancer, reflecting the less integrated, more "foreign" nature of the viral genetic material, which may lead to a peculiar tendency to interfere with normal regulation.

Viruses are the migratory components of the cellular genome. As such, they offer unique opportunities for experimentation and for analysis of integrative patterns between genetic elements. They afford new insights into the functional organization of cells. Even though their role as etiological agents of cancer may prove to be a rare one, they still deserve the place they have recently assumed—at the center of the stage of cancer research.

RESUMEN

Los virus pueden producir alteraciones celulares permanentes de dos modos: ya sea iniciando nuevos procesos de síntesis dirigidos por los genes virales o estableciendo nuevos controles reguladores sobre la función de los genes celulares. Se presentan ejemplos del primer tipo de mecanismo, derivados del estudio tanto de virus bactéricos como de virus animales. Ejemplos posibles del segundo tipo de mecanismo también se discuten. Se señala que las manifestaciones de la función dirigida por virus dependen a menudo del genotipo y del estado fisiológico y de desarrollo de las células del huésped. El papel de las alteraciones celulares inducidas por virus para determinar la respuesta de la células a los factores ambientales es examinado. Las implicaciones de estos fenómenos con el problema de la carcinogénesis son expuestas.

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DISCUSSION

Mazia: * Would you predict that if those of us working with DNA polymerase isolated good DNA from brain this DNA would not prime?

Luria: I think that the experiment has already been done; the answer so far as I know is what one would have expected: the DNA is a perfectly good primer. The meaning of this is not clear, however. In bacterial cells, transforming DNA is not replicated until it is incorporated into the cell genome, but it is not broken down. *In vitro* this is a good primer. Perhaps the Kornberg enzyme is only a part of the whole machinery needed to replicate DNA. To make a good primer *in vivo* you may have to have, for example, an unzipping enzyme or some sort of phosphatase that makes an initiation point.

It is possible that differentiated cells do not divide, because some necessary factor is missing or inhibited. But I am rather excited by the fact that the host-induced modification observed in viruses for the first time has revealed a new level of structural changes in nucleic acid, which, without changing the genetic information, controls the range of cells in which the DNA can reproduce. It would be surprising if such a mechanism of regulation would not be used. Of course, it may have nothing to do with differentiation. It may be a mechanism of genetic isolation, which prevents extraneous DNA from being duplicated in a cell.

Rogers: Dr. Luria's points are extremely pertinent to the tumor problem. For example, in the work of Shope there is almost a precise model of what may be this same thing found with the Shope papilloma virus. The virus derived from the wild rabbit, generally called the wild type, is nonrecoverable from domestic rabbit tumors. There is another line of virus which Shope has discovered which is recoverable from domestic rabbit tumors. If you put the recoverable line of virus back into the wild rabbit it always reverts to the wild type and is no longer recoverable in the domestic line of animals. This rather remarkably resembles what Dr. Luria describes. In addition, we have found that domestic rabbit tumors induced with wild-type virus contains an antigen that reacts as a wild rabbit antigen, and conversely wild rabbit tumors induced with the recoverable line of virus contain a domestic rabbit antigen. No cross-reaction has been found between these two antigens.

Luria: In 1953 I pointed out the similarity between the Shope rabbit papilloma situation and host-induced modification.

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Shope Papilloma Virus: Concerning Its Relation to Cell Division¹

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SUMMARY

Literature relating to operational genetic information carried by virus genomes is briefly reviewed, particularly the previously reported findings with the Shope rabbit papilloma virus. That viruses may act through direct (Shope papilloma virus) or indirect mechanisms (the polyoma and Bittner milk agent) is discussed. Evidence is presented on the metabolic difference between the nonrecoverable and recoverable virus lines of Shope, which, in addition, suggests that the virus information accounting for nonrecoverability in the domestic rabbit is owing to transduced wild rabbit in-

formation relating to serine metabolism. It was pointed out that if a line of rabbits with a genetic deficiency and unable to synthesize arginase was available, the genetic information to degrade arginine to ornithine and urea could be given them through infection with the papilloma virus, as animals carrying the papillomas have an arginase derived from virus information and further have a low blood arginine. The utilization of passenger viruses to supply genetically deleted information in man seems thereby indicated.—Nat Cancer Inst Monogr 14: 107-115, 1964.

IT HAS LONG been recognized that for a virus to cause a tumor it must in some way heritably modify the growth or death rate of the infected cells. Looking at the literature retrospectively, it would appear that one of the earliest indications that viruses were doing more in cells than simply replicating and thereby modifying the metabolism and growth of the cells was the discovery by Rous that the resistance directed toward the agent *per se* is distinct from the resistance manifest against the alien tumor cells in fowls implanted with virus-induced chicken tumors (1). There was then a gap of many years before Kidd discovered the Vx-2 antigen in a transplantable cancer arising in a Shope virus-induced papilloma of rabbits (2). As sufficiently inbred chickens or rabbits were not available in either of these examples, the doubt remained that these antigens were not necessarily the results of a specific change brought about directly by the virus. However, the discovery of the Vx-7 antigen in another cancer, the Vx-7, appearing in a Shope virus-induced papilloma

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made a direct relation more likely (3). Other information indicating that replication of the virus *per se* was not the critical factor in virus disease was supplied shortly thereafter by Sabin who reported that two variants of yellow fever virus replicating at essentially the same rate differed widely in their pathogenicity (4). A breakthrough was the discovery of a new and unique pyrimidine, 5-hydroxymethyl cytosine, in virus-infected *Escherichia coli*, by Wyatt and Cohen (5). The brilliant biochemical exploitation of this finding (6) and other related findings in a number of laboratories indicate that viruses bring in with their DNA (7) genetic information relating to metabolic function not manifest in the original host genome. It is thus evident that to understand virus-associated biological phenomena and certainly to treat those disease-associated, we must determine the genetic information brought in by individual virus and its interaction with the host genome. The magnitude of this problem as well as the variety of interactions which may take place between the virus and host genome are well covered in a recent review by Luria (8).

At present the two general approaches to this problem using animal viruses are immunologic and metabolic. After an immunologic gap of almost 15 years from Kidd's work, Zilber demonstrated an abnormal antigen in the Rous sarcoma (9). It is perhaps fitting that this finding was in the Rous sarcoma, occurring almost 50 years after Rous described an antigen in addition to the virus in a virus-induced tumor. In any event, it has stimulated a great flurry of highly productive work by a variety of investigators. Virus-specific antigens have now been described for the Rous sarcoma (9), Shope papilloma (2, 10, 11), SV40 and polyoma tumors (12-14), Adenovirus 12 tumors (15), Gross leukemia (16), and Moloney virus leukemia (17).

Our area of interest has involved primarily metabolic approaches as to how a virus works, though at times immunologic methods have proved useful. The basic method used is quite a simple one: a roughly quantitative comparison of the virus-infected tumor cells with their normal or hyperplastic counterpart (18-20).

These comparisons relate to the metabolite concentrations in the cells and on the metabolite utilization by the cells while incubated in a synthetic media of known composition. These studies were done with the assumption that any new metabolically significant information brought in by the virus, or induced in some way by the virus in the infected cells, would be reflected by detectable changes in the metabolism of the cells and that once a difference, preferably qualitative, was found its biological significance would be determined for cell operation and genetic source of information.

The Shope virus-induced rabbit papilloma seemed ideal for these studies because of the broad biological background made known by the extensive and excellent work of Shope and Rous and associates, and equally critical, the ready availability of the normal and normal hyperplastic counterpart to the virus-infected papilloma cells. In comparisons of the amino acid metabolism of normal and virus-infected cells, we were fortu-

nate in encountering a qualitative difference for arginine metabolism. This difference concerned the presence of an arginase in the virus-infected cells not demonstrable in the normal counterpart (20). No other enzymes of the arginine-urea cycle were found. With C¹⁴-arginine it was found that almost all the arginine taken up by the cells was rapidly excreted as urea and ornithine (10). Histochemical studies were done because of the work of Allfrey and Mirsky, which indicated that nuclear synthetic activity was controlled by arginine-rich histones (21). The almost complete depletion of arginine-rich histones in the nuclei of the virus-infected cells as opposed to their normal or hyperplastic counterparts was most striking (10). It was then determined to find whether this enzyme, induced in such large quantity in the virus-infected cells, was induced in a way analogous to the induction by chemicals (22), that is, from rabbit genetic information, or whether its induction was like that of bacteriophage where the enzyme responsible for the existence of 5-hydroxymethyl cytosine may be derived from virus information (6). The immunologic approach seemed uniquely suitable for this purpose as it had been long known that animals carrying these papillomas developed antibodies against the virus (23). Should the arginase be derived from virus DNA information, one would expect that rabbits carrying the papillomas would also have antibodies against the arginase; the enzyme was therefore purified. Specific precipitins were found. The sera did not cross-react with either purified domestic or wild rabbit liver arginase, the antibodies were not absorbed out by large amounts of purified virus, and the sera from either wild or domestic rabbits carrying papillomas were equally effective against the purified arginase derived from either wild or domestic rabbit papillomas (10). These experiments provided critical evidence that the animals carrying these virus-induced papillomas handled this enzyme derived from their own tumors as a foreign protein. As papillomas induced with purified virus contain a high concentration of this same enzyme, and since it has been demonstrated that virus DNA is responsible for the neoplastic state of the cells (24), it follows that the information responsible for the existence of the enzyme is derived from virus DNA. Analytical studies of the purified enzyme further substantiate these findings. Papilloma arginase is, in every parameter tested, remarkably different from rabbit liver arginase, kidney arginase, an argainse in a tar-induced carcinomatoid of rabbit skin or any previously described enzyme of this sort (25). Perhaps one of the most interesting differences is that papilloma arginase, in contrast to all the others, does not require divalent ions such as manganese or cobalt for activation. Instead it is most active in water and even in the presence of ethylenediaminetetraacetate (EDTA) which acts to bind even trace amounts of divalent ions which might have been around after extensive dialysis. It is more important, however, to the question of whether the enzyme is derived from rabbit or virus genetic information, that the peptide patterns of enzyme derived from either wild or domestic rabbit papillomas are alike and in marked contrast to the patterns ob-

tained with purified liver arginase. The peptide patterns of domestic rabbit and wild rabbit arginase were quite perceptibly different, as expected from previous comparisons of this sort. Such species differences have been the rule and point up the significance of the fact that the patterns of wild and domestic rabbit papilloma arginase are alike (25).

It next seemed pertinent to find whether the virus-induced arginase could be bypassed, for if it could, the growth of the tumors should be greatly slowed as more arginine would be available for nuclear histone synthesis. A number of experiments was tried using dietary and parenteral supplementation with arginine but it was not possible by this method alone to raise the blood level of the amino acid. This had been known for some time from the work of Womack and Rose (26). It was found, however, if small amounts of the arginine antagonist, canavanine, were given in addition that the blood level could be raised and the growth of the tumors brought almost to a standstill. The close relation of these findings with the papilloma to the work of Allfrey and Mirsky and their findings of the repressor effect on nuclear synthetic activity particularly messenger RNA synthesis (22, 27) is thereby further substantiated (25). It is particularly noteworthy that with another virus system, the Ehrlich ascites carcinoma-Bunyaamwera system, where a tremendous increase in the amount of arginase was found in the virus-infected cells, Colter, Bird, and Koprowski found the amount of protein-bound arginine greatly reduced (28). Whether this reduction was primarily from a depletion of arginine-rich histones is not known; neither is information available as to whether the enzyme induced is of the host cells or the virus. Also worthy of mention is the acceleration in the mitotic rate of grasshopper neuroblasts by agmatine, probably an arginine antagonist (29).

What other information is on the Shope virus DNA? Certainly the presumed subunit of the protein coat. In addition, a wild rabbit antigen, distinct from the virus or arginase, has been found in domestic rabbit papillomas induced with purified Shope virus obtained from papillomas of the wild cottontail (25). This antigen has raised the question as to the actual size of the Shope virus DNA as apparently wild rabbit information is being brought along with the virus DNA package. Two other antigens are known to be associated with the Shope virus infection: 1) the Vx-2 antigen found in two transplantable carcinomas arising in Shope papillomas (2), and 2) Evans' antigen related somehow to sporadic retrogression of the growths (11). There is no evidence whether either of these two latter antigens is derived synthetically from virus information.

There are two lines of Shope papilloma virus: 1) the wild type not usually recoverable in the domestic rabbit and then, in exceedingly small amounts, and 2) the recoverable line which may be readily passed in the domestic rabbit (30). On comparison of the metabolic characteristics of the papillomas induced in domestic rabbits the arginase is similar in both. There is, however, a large quantitative difference in the amount of serine utilized by the two tumor types which, incidentally, are morphologically identical. Papillomas induced with wild-type virus use large

amounts of serine in contrast to those induced with the recoverable line (31). It was quite interesting to find that normal wild rabbit squamous epithelium uses large amounts of serine in contrast to normal domestic rabbit epithelium. Serine supplementation of the diet by parenteral injection greatly increases virus recoverability in the wild rabbit; however, as yet it has not been possible to make the wild-type virus recoverable in the domestic rabbit with serine (31). A particularly important report is that when the recoverable virus line is passed through wild rabbits it reverts to wild type (32). Studies are under way to find whether this reversion is always associated with a change of serine metabolism in the domestic rabbit papillomas, as this rather remarkably parallels the findings of Morse, Lederberg, and Lederberg (33) with transduction with bacteriophage. In other experiments, with the papillomas induced in wild rabbits with the recoverable line of virus obtained from domestic rabbits, a domestic rabbit antigen has been found in the wild rabbit tumors. However, this experiment has not yet been done with purified recoverable virus so a chance remains for explanations other than transduction.

Is the polyoma virus acting in other ways like the papilloma virus? It has been suggested in a number of laboratories that since the DNA's of the two viruses are roughly the same size and have the same melting characteristics that they might have much in common (34). However, there are some great differences. For example, the latent period to neoplastic transformation following infection of cells with the polyoma virus is quite long and in culture systems during this latent period the incidence of chromosome breakage is greatly increased (35). Furthermore, all the known antigens associated with polyoma virus infection are found early after infection with the virus (36). These facts together appear to indicate that the information of the polyoma virus DNA is not directly associated with the neoplastic transformation and further raise the question as to whether we should classify the polyoma virus as a tumor virus at all since the neoplastic transformation seems incidental to its presence. This is all in marked contrast to the papilloma virus which results in an immediate transformation of the cells to tumor cells, and, as Rous has phrased it, is the *actuating* cause of the neoplasm (37). Another virus which seems to be acting like the polyoma virus is the Bittner agent. Its long latent period, the associated non-neoplastic hyperplastic plaques (38), and the finding that the breast cancers arising in virus-infected mice are almost entirely hormone-dependent (39), suggest that the action of the Bittner agent is simply to increase the sensitivity of the infected breast cells to mammotropin and, like polyoma, the information of the virus nucleic acid has no direct relation to the neoplasms occurring.

The occurrence of the phenomenon of having viruses only very indirectly related to the occurrence of neoplasia such as polyoma and the Bittner agent is immensely important to the directions we take in relation to the therapy of neoplasia in man. Obviously, we must understand the metabolic aberrations in the individual neoplasm if we are to bypass excesses,

substitute deficiencies, or do much more than palliate the disease with metabolic antagonists.

On the other hand, viruses offer an extremely bright future for the control of the host genome, not only because of the genetic information they carry themselves but through transductive possibilities. For example, if there were a rabbit line available in which there had occurred a deletion in the genetic information for the synthesis of arginase, one could quite readily give the animals an arginase by infecting them with the Shope virus. That the enzyme would be in epithelial cells rather than liver and kidney cells might well prove not a problem for we have found that the Shope virus has a systemic effect upon the blood arginine. It tends to run relatively low. We do not know as yet whether this is wholly the result of the high utilization of arginine by the papillomas. Of course, giving an animal a tumor to relieve a genetic deficiency would not appear very realistic therapy. Yet, we know many passenger viruses which have no discernible effects, such as herpes, most of the time. With an organized search we might well be able to find a passenger virus carrying the information for the synthesis of phenylalanine hydroxylase. Would not this be a boon to the phenylketonuric? There is strong evidence that certain bacteriophages carry information for the synthesis of thyminesynthetase, and cytosine hydroxymethylase (6) and that vaccinia induces thymidine kinase in tissue-cultured cells lacking this enzyme (40). The possibilities for the future with the techniques available to us today are indeed immense.

RESUMEN

La literatura relativa a la información genética operacional transportada por la suma de genes virales es brevemente revisada, en particular los hallazgos previamente referidos con el virus del papiloma Shope del conejo. Que los virus pueden actuar a través de mecanismos directos (virus del papiloma Shope), ó indirectos (el virus del polioma y el agente leche de Bittner) se discute. Se presenta evidencia sobre la diferencia metabólica entre las líneas de virus Shope no-recuperables y recuperables, lo cual sugiere que la información viral que motiva la no-recuperabilidad en el conejo doméstico es debida a la información del conejo salvaje transducida en relación con el metabolismo de la serina.

Se señala que si pudiera obtenerse una línea de conejos que tuviera una deficiencia genética y fuera incapaz de sintetizar arginasa, la información genética para degradar la arginina á ornitina y úrea podría serles dada mediante la infección de los conejos con el virus del papiloma, ya que los animales portadores de papilomas tienen una arginasa derivada de la información viral y tienen una baja arginina en la sangre. La utilización de los virus pasajeros para proveer la información suprimida genéticamente en el hombre parece por lo tanto estar indicada.

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DISCUSSION

Rabson: In regard to the rapidity of oncogenic activity of polyoma virus, Michael Stoker (Brit J Cancer 14: 679-689, 1960) has presented evidence that hamster cells *in vivo* may be transformed to tumor cells in less than 24 hours after inoculation of the virus. He has demonstrated foci of tumor cells in the kidneys of hamsters 4 days after inoculation of virus; and, by counting the cells in the foci and assuming a cell division time of approximately 16 hours, it can be shown that the tumor must have been initi-

ated shortly after injection of virus. This would compare favorably with rapidity of action of the Shope papilloma virus.

There has been some question about the presence of arginase in normal rabbit skin. Will you comment on the work of Rothberg and Van Scott at the National Institutes of Health?

Rogers: There was a report in *Nature* 2 or 3 years ago, apparently of some small amount of arginase in the skin of rabbits. A homogenization system that was lyophilized was used, and contained from 5 to 10 million bacteria per cc of test material. It was not clear whether or not the arginase that they found was bacterial or was, indeed, a rabbit arginase. The arginase they described is manganese-dependent in contrast to the papilloma arginase, which works best in the distilled water.

Luria: I think the main elements of your paper are contained in your statement that you were fortunate to find in these papillomas an enzyme which, from all the evidence, appears to be controlled by the virus, and which, in this case, has some hopeful possibility of being related to the mechanism of cellular differentiation and, therefore, carcinogenesis. Those of you who heard my discussion, and especially the discussion after my paper in Lima, will remember that I pointed out how careful one has to be before deciding that there is a causal relation between any change, antigenic or other, that may be found in cells infected with a virus, and the tumoral properties of the cell. In Dr. Rogers' case the involvement of arginase and of levels of nucleohistones suggest that the findings may be relevant to the tumoral property.

I have been thinking that polyoma and papilloma viruses which are similar in many ways, may also have in common some functions in the cell. Has a search been made for similar types of arginase in polyoma-induced tumors? In this respect, it may be interesting to look at other properties of tumor arginases that may make them characteristic, such as heat sensitivity, sensitivity to inhibitors, etc.

One other thing I would like to mention as a possible suggestion is the following: As Dr. Rogers has discussed, in the normal skin only the basal layer cells can divide, whereas the cells removed from the underlying derma stop dividing. In the tumor there is continued division in layers of cells further removed from the dermal surface. One may think that some substance which acts as a stimulus to the initiation of DNA synthesis may normally come from the derma, and that the cells of the basal layer may have a certain susceptibility to its action, and cells in more distant layers may receive less and less of this substance. In the case of the papilloma there may be, for example, a lower threshold for cellular response to the stimulus to replication which comes from the derma, or, more likely, a higher permeability to it.

Rogers: In relation to Dr. Luria's question concerning the possibility of similar types of arginase being in polyoma virus-inducing tumors, we have never tested these. The test was made recently on some adeno-12 virus hamster tumor material of Drs. Rouse and Schlesinger and only trace amounts were found, these amounts being readily explainable by contaminating cell types.

Dr. Luria's suggestion that there might be something in the papilloma accounting for a lower threshold for cellular response to the stimulus to replication, or should the stimulus come from the dermis, a higher permeability to it, is an interesting one. Though we have no evidence for it, there is no reason to believe that the virus might not be carrying information for a permease or somehow inducing one.

Stem Cell Renewal Systems and Radiation Effects

Classification of Cell Populations on the Basis of Their Proliferative Behavior^{1, 2}

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SUMMARY

The increase in the number of nuclei with age was estimated from the deoxyribonucleic acid (DNA) content of organs and tissues of young, growing rats and expressed as the *daily nuclear addition rate*. A search was also made for dividing cells, especially in radioautographs after H^3 -thymidine injection and after administration of colchicine. The use of this drug made it possible to measure the *daily mitotic rate*. Preliminary results had suggested that the cell populations of the embryo increase exponentially, but, toward the time of birth, exponential growth ceases. The cell populations were then investigated with the use of rats ranging in age from about 7 to 90 days. In some populations, no mitotic activity was detected and the DNA content did not change. Hence, the number of cells remained constant during the period investigated. These cell populations are referred to as *static*, e.g., neurons. In other organs and tissues, the DNA content and, therefore, the number of cells kept increasing, though at a slower and slower rate with age. This increase

could occur in two ways. In the first case, mitoses were scattered and their number accounted for the increase in DNA content (and, therefore, for the addition of nuclei). Hence, all cells added by mitosis were retained. These cell populations are referred to as *expanding*, e.g., parenchymal cells of liver and kidney, muscle fibers, etc. In the other case, mitoses were abundant and their number greatly exceeded that required for the increase in DNA content (and, therefore, many more cells were produced than were retained *in situ*). Cell losses must have taken place, in such a manner that the high cell production only slightly exceeded the losses. These cell populations are referred to as *renewing*, e.g., cells of epidermis, intestinal epithelium, thymus, etc. In *neoplastic* cell populations, proliferation of the cells and their progeny tended to continue indefinitely. Even, if some cells stopped dividing and some degree of renewal occurred, cell production greatly exceeded the cell losses.—Nat Cancer Inst Monogr 14: 119-150, 1964.

THE EGG CELL and its progeny divide repeatedly to give rise to the variety of specialized cells making up the adult organism. It is commonly believed that as cells become specialized or, to use the accepted term, "differentiate," they lose the ability to divide. Thus Rusch (1)

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³ The author is indebted to Dr. M. Enesco who is responsible for much of the material used.

proposed a model of the evolution of cell populations, according to which increasing differentiation is associated with a decrease and eventually a loss of the ability to divide.

According to this model, the cells that keep on dividing actively throughout life (2, 3) should be little or not differentiated.⁴ However, when the proliferative behavior of cell populations was examined in young rats, this conclusion was found to be true in some but not in all cases. The differences led us to group cell populations into three classes: static, expanding, and renewing. The present article defines these three classes and describes their proliferative behavior.

When Rusch presented his model he also stated that, whereas the fully differentiated cells of the adult do not divide, neoplastic cells duplicate themselves indefinitely (1). In guise of conclusion, the behavior of our three classes of cell populations will be compared briefly to that of neoplastic cell populations.

METHODS

The proliferative behavior of cells was investigated in two ways. First, the deoxyribonucleic acid (DNA) content of various organs and tissues was measured in rats of various ages by M. Enesco to find out if the number of nuclei changed with age; when this was so, the rate of change was estimated. Second, a search was made for dividing cells in histological sections, as well as in radioautographs following injection of H^3 -thymidine. When mitotic activity was present, the mitotic rate was usually measured with colchicine.

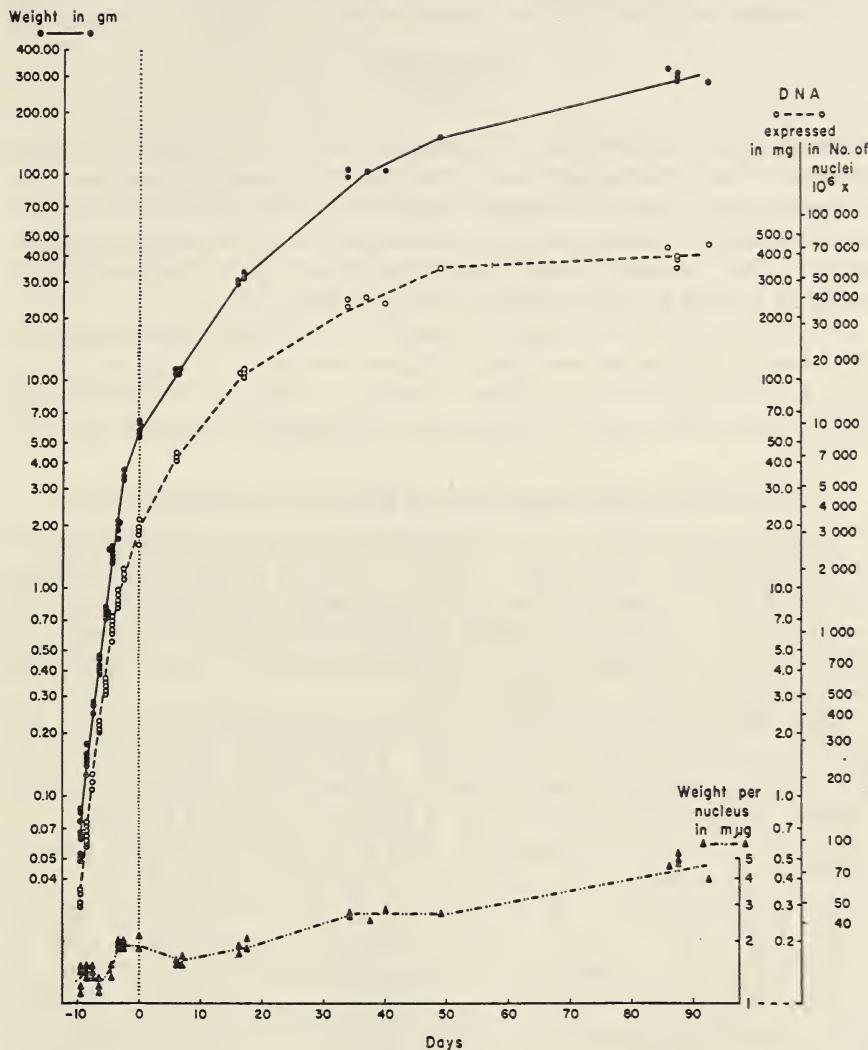
Estimation of the Number of Nuclei From DNA Determinations (M. Enesco and Associates)

Since the nuclei in the various organs of the rat have the same DNA content (with a few exceptions, *e.g.*, some liver nuclei), the number of nuclei in any one organ may be obtained by dividing the total DNA content by the amount of DNA in one nucleus, $6.2 \mu\text{g}$ (4). Since there is one nucleus per cell in most organs of young rats, the number of nuclei is usually equal to the number of cells.

An idea of the possibilities of the technique may be obtained from measurements of the number of nuclei in the whole body of male rats of various ages, as shown in text-figure 1. Thus, the number of nuclei was

⁴ Cowdry (2) has proposed an interesting classification of cells according to their ability to proliferate. Of the cells which divide, some do so repeatedly (vegetative intermitotics) and others a limited number of times only (differentiating intermitotics). Of the cells which do not divide, some retain the possibility to do so under special conditions (reverting postmitotics) but others do not (fixed postmitotics). Cowdry (2) has used this classification rather rigidly. Thus vegetative intermitotics include an ill-assorted group: embryonic cells, stem cells of epidermis and blood-forming organs, neoplastic cells, etc. An important feature of the classification is that a cell beginning its life as an intermitotic may become a differentiating intermitotic and later a fixed postmitotic (epidermis). In other words, cells are considered at a given moment of their life rather than as members of a population.

WHOLE BODY



TEXT-FIGURE 1.—*Whole body*. Semilogarithmic plot of the weight in grams (●), DNA in mg (○), and weight per nucleus in m μ g (▲) of male rats *versus* the age in days. The DNA is expressed in mg, and also converted into millions of nuclei (scale at right). The time of birth is indicated by the *vertical dotted line* at 0 days. The plot of the log of the *number of nuclei* *versus* time approximates a straight line almost until birth, which suggests that embryonic cell populations grow more or less exponentially. Later, the curve rises less and less steeply as the animals become older. The mean *weight per nucleus* increases from 7 to 90 days after birth, due to cell enlargement and accumulation of intercellular material. [Reproduced from (4).]

estimated at 50 million in the youngest embryos examined (10 days before birth), 3 billion at birth, and 67 billion in 90-day-old rats (4).

The rate at which the number of nuclei increases per day, referred to as *daily nuclear addition rate*, was measured by the formula of Brody (5),

$$\frac{\ln N_2 - \ln N_1}{t_2 - t_1},$$

where $\ln N_1$ and $\ln N_2$, respectively, stand for the natural logarithms of the number of nuclei at ages t_1 and t_2 in days. In practice, the rate was measured over a period extending between two estimations of the number of nuclei, and the result was expressed as percent of the mean number of nuclei. For instance, the daily nuclear addition rate for the whole body was 0.25 percent per day between 40 and 90 days.

The DNA method also makes it possible to calculate a mean figure for the amount of material associated with one nucleus, or *weight per nucleus* (organ weight divided by number of nuclei). This index increases when cells enlarge and/or when the proportion of intercellular material rises (4).

Identification of Dividing Cells and Measurement of Mitotic Rate

DNA determinations do not distinguish between the various types of nuclei present in a structure. Usually, we have to deal with at least two cell types: 1) parenchymal cells characteristic of the organ or tissue under study, *e.g.*, follicular cells of thyroid, acinar cells of pancreas, and muscle fibers of skeletal muscle; 2) supporting cells, *e.g.*, cells from connective tissue, nerves, and blood vessels.

Examination of the histological section of a structure may or may not reveal the nature of the dividing cells. Furthermore, the rate at which the divisions take place can seldom be deduced from straight counts of mitoses, because the exact duration of these mitoses is not known.

Mitotic rates may be calculated after injection of a suitable dose of colchicine or of derivatives such as Colcemid, which stop dividing cells at the metaphase stage. Stevens Hooper (6) showed that at certain dose levels, and within a certain period, colchicine arrests dividing cells in metaphase without interfering with the normal rate of entry of cells into mitosis. Therefore, colchicine may be used to measure the number of cells undergoing mitosis over a certain period, say 4 or 6 hours, and from these data the mitotic rate may be calculated. This rate has been measured in the intestinal epithelium of rats (7) and cats (8), the plantar epidermis of rats (9), the alveolar cells of the rat lung (10), and other tissues. The procedure is as follows (9-11): Four groups of rats are killed 6 hours after colchicine injection; 1 group each at night, in the morning, in the afternoon, and in the evening, with a 6-hour interval between the killing of any 2 consecutive groups. Hence, a group is killed at the time the next group receives colchicine. For each organ, the percent of cells arrested in metaphase in the 4 groups are added to obtain the total percent of cells entering mitosis over a 24-hour period,

that is, the *daily mitotic rate*. This method was used by Bertalanffy to obtain the data on the kidney, adrenal, and lung, and by MacConnachie to obtain the data on skeletal muscle. MacConnachie used six 4-hour intervals.

With the help of radioautography, the use of the DNA precursor, H^3 -thymidine, has allowed a different approach. A cell which is in the process of doubling its DNA prior to mitosis takes up injected H^3 -thymidine into its nucleus. If radioautographs are prepared from organs or tissues obtained soon after injection, the H^3 -containing nucleus is overlaid by a collection of silver grains. Later, the labeled cells are seen to undergo mitosis (text-fig. 6), at the end of which both daughter cells are in turn labeled (12). Thus, a dividing cell may be identified *before* and *after* the division takes place. Both possibilities were utilized in the work now to be described.

RESULTS AND DISCUSSION

Characters of Embryonic Cell Populations

Within the 22 to 23 days of the rat's embryonic life, the egg cell and its progeny divide many times to give rise to the organs and tissues of the newborn. Our earliest DNA estimations (4) made 10 days before birth, that is, 12 to 13 days after fertilization, revealed that some 50 million cells were present at that time (text-fig. 1). If it is assumed that all cells divide repeatedly and regularly and no cell loss occurs, then it would take 25 to 26 generations for the egg to produce this number of cells over the 12- to 13-day period. Hence, during that time the number of cells would double every half day on the average. Such a rapid growth implies a high daily nuclear addition rate of well over 100 percent.

If, for the last 10 days of embryonic life, we again assume regular division of all cells and no loss, it would take only 6 generations for the 50 million cells present 10 days before birth to yield the 3 billion cells present at birth. Therefore, cell division must be less frequent than in the early period. Furthermore in text-figure 1 the plot, log nuclear number *versus* time, is a fairly straight line almost until birth and, therefore, the number of nuclei increases exponentially during most of the period, so that the daily nuclear addition rate must be fairly constant. This rate averaged 60 percent per day (doubling time of the cell population, 1.7 days). This is still a rapid rate, though somewhat slower than in the early period. The decrease could be due in part to the emergence of cells which no longer divide. Indeed, H^3 -thymidine data from 16-day rat embryos revealed that a few cells, *e.g.*, ganglionic cells, have stopped dividing at that time. But the vast majority of embryonic cells keep on proliferating (13). Hence, the main explanation for the decrease in rate of nuclear addition must be that the mitoses succeed each other less frequently than in the early period.

For convenience, cell populations were considered "embryonic" until the time of birth. In the period immediately before and soon after birth, the cells of most organs (*e.g.*, pancreas, liver, and cerebellum) and tissues (*e.g.*, muscle and cartilage) acquire characteristic cytological features and presumably become functional, that is, they differentiate. Since it was believed that by 7 days after birth cells were well differentiated, our main work was started in rats of that age (4). And yet, much cell proliferation must take place before the 7 billion cells of the tiny 11 g rat pup of 7 days give rise to the 67 billion cells of the large 300 g rat of about 90 days. The observations made during that period of growth (7 to 90 days) led to the classification of cell populations as static, expanding, and renewing.

Characters of Cell Populations in the Postnatal Rat

A cell population was defined in the postnatal rat as a group of cytologically similar cells presumably with a similar function, *e.g.*, the acinar cells of pancreas. The term may be restricted to a definite cell type, such as the Purkinje cells of the cerebellum; or it may include a series of related cell types, *e.g.*, all neurons; or it may refer to all the cells coming from certain stem cells, *e.g.*, Malpighian cells of the epidermis, seminal cells of testis.

Static Cell Populations

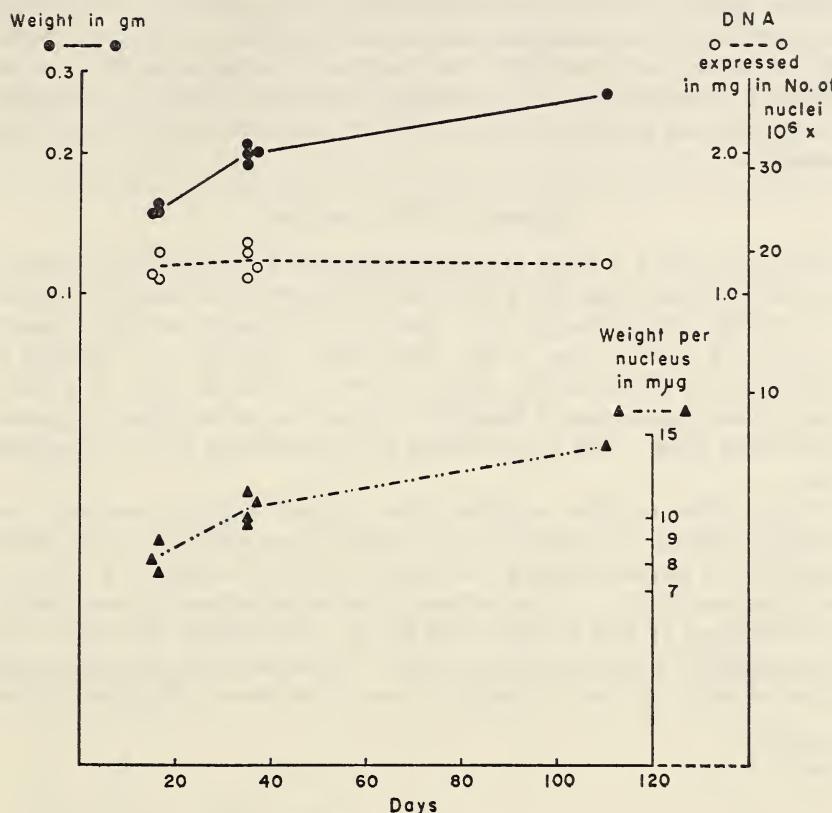
The consensus is that within a few days after birth the number of nuclei becomes constant in the nervous system (14), retina (2), and muscle tissue (15). If this is correct, the postnatal growth of these structures would be due to increases in cell size without change in cell number.

In preliminary experiments of Enesco (16), designed to check the accuracy of this conclusion, the DNA content was measured in cerebrum, cerebellum, and spinal cord in young rats of various ages. The only one of these regions that so far has shown constancy of the DNA content with increasing age is the cerebellum (text-fig. 2).

The DNA constancy in cerebellum suggested that its cells do not divide. If so, no colchicine-arrested metaphases or H^3 -thymidine-labeled cells should be present. This was indeed true with the neurons of cerebellum, as well as with the neurons found in other parts of the central nervous system and ganglia in young adult mice (17). The possibility that the odd ganglionic cell might be labeled in the myenteric plexus of rats (12) prompted us to set up an experiment in which H^3 -thymidine was injected every 3 hours into 16 g rats over a 6-day period (18). Neither central nor peripheral neurons were labeled under these conditions so it was concluded that neurons of young and adult rats and mice do not divide after the age of 7 days.

Static cell populations were then defined in 7- to 90-day-old rats as homogeneous groups of cells in which no mitotic activity can be detected even with colchicine or H^3 -thymidine, so that the total DNA content remains constant.

CEREBELLUM



TEXT-FIGURE 2.—*Cerebellum*: The weight increases slowly with age, but the number of nuclei shows no significant change between the ages of 17 and 110 days. The weight per nucleus increases with age, thus accounting for the over-all weight increase. ● = Weight in g; ○ = DNA content in mg (or millions of nuclei); ▲ = weight per nucleus in m μ g (16). The same symbols apply to text-figures 3, 4, 5, 7, 8, 9, and 10.

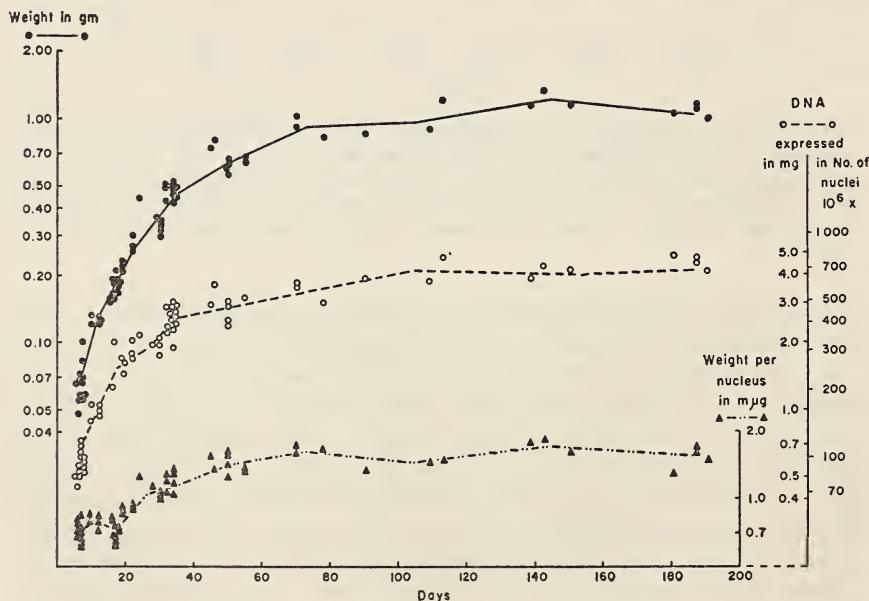
Not all cells of the nervous system belong to static populations. Thus, after injection of H^3 -thymidine, the label may be found in: 1) some neuroglia cells of an immature type referred to as spongioblasts (17), though the frequency of labeling decreases rapidly with age in rats and mice (19); 2) some cells of the subependymal layer, a region located immediately below the ependyma in various regions of the third and lateral ventricle (20). In some parts of the brain, the divisions taking place in these two cell populations might add enough new cells to cause an increase in DNA content with age. Further work must be done to find out whether, besides neurons, there are other static cell populations in the nervous system. (The retina should also be further investigated. As for the various types of muscles, they are not composed of static cell populations, as will be shown in detail for skeletal muscle.)

In the cerebellum, labeled cells were rare, so that any increase in cell number would be quite small and unlikely to be detected by DNA determinations. The substantial increase in the size of cerebellum during the postnatal period (text-fig. 2) is therefore not due to proliferation of cells, but to an increase in the "weight per nucleus," which in the cerebellum implies an increase in both cell size and amount of intercellular material.

Expanding Cell Populations

When the DNA content of various organs and tissues of the male rat was plotted against age (4), a progressive increase in the number of nuclei was usually found, though with a gradually decreasing rate (text-figs. 3, 4, 5, 12, 13, and 14), as in the whole body (text-fig. 1). The use of colchicine and H^3 -thymidine showed, however, that in spite of a somewhat similar appearance of the DNA curves, two quite different types of populations exist. The first, referred to as *expanding*, will be considered now.

Organs.—Let us first examine three organs: kidney, pancreas, and adrenal. Between 7 and about 90 days of age, the number of nuclei increased 6.5 times in kidney (text-fig. 3) and even more, 18.2 times, in pancreas (text-fig. 4). In the adrenal, the number of nuclei increased 3.7 times between 17 and 90 days (text-fig. 5). The figures for kidney and adrenal may be read as number of cells. In the pancreas, binucleate cells appeared with age and, therefore, there were fewer cells than nuclei,

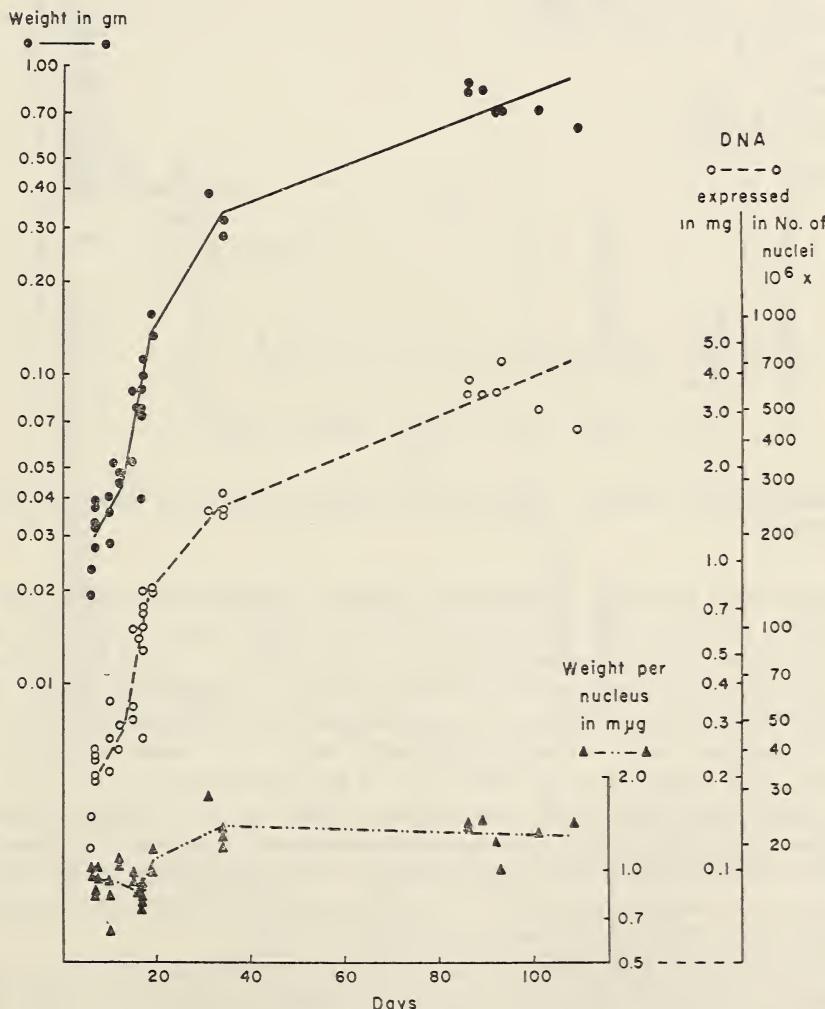


TEXT-FIGURE 3.—*Kidney.* The curves for weight and number of nuclei rise, but at a progressively decreasing rate. The weight per nucleus increases significantly between 17 and 34 days, due to increase in cell size at that time (4).

but even if all cells had become binucleate, which is far from being true, there would still be a pronounced increase, 9.1 times, in cell number. (There was also a moderate, early cell enlargement in these organs, as suggested by increases in the weight per nucleus of 2.1, 1.2, and 1.4 times in kidney, pancreas, and adrenal, respectively. *See text-figs. 3, 4, and 5.*)

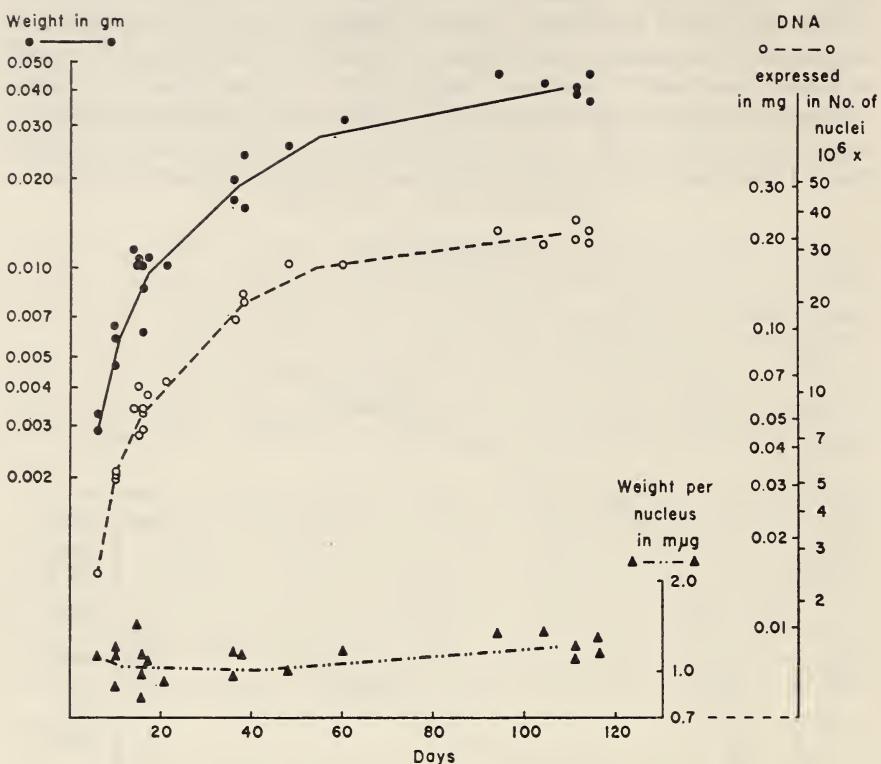
The next step was to find out if the added cells were parenchymal or supporting cells or both. Radioautography after H^3 -thymidine injection showed the label in cells of the two types, indicating that both proliferate.

PANCREAS



TEXT-FIGURE 4.—*Pancreas.* The curves for weight and number of nuclei rise with age. The weight per nucleus goes up between 17 and 34 days only (4).

ADRENAL

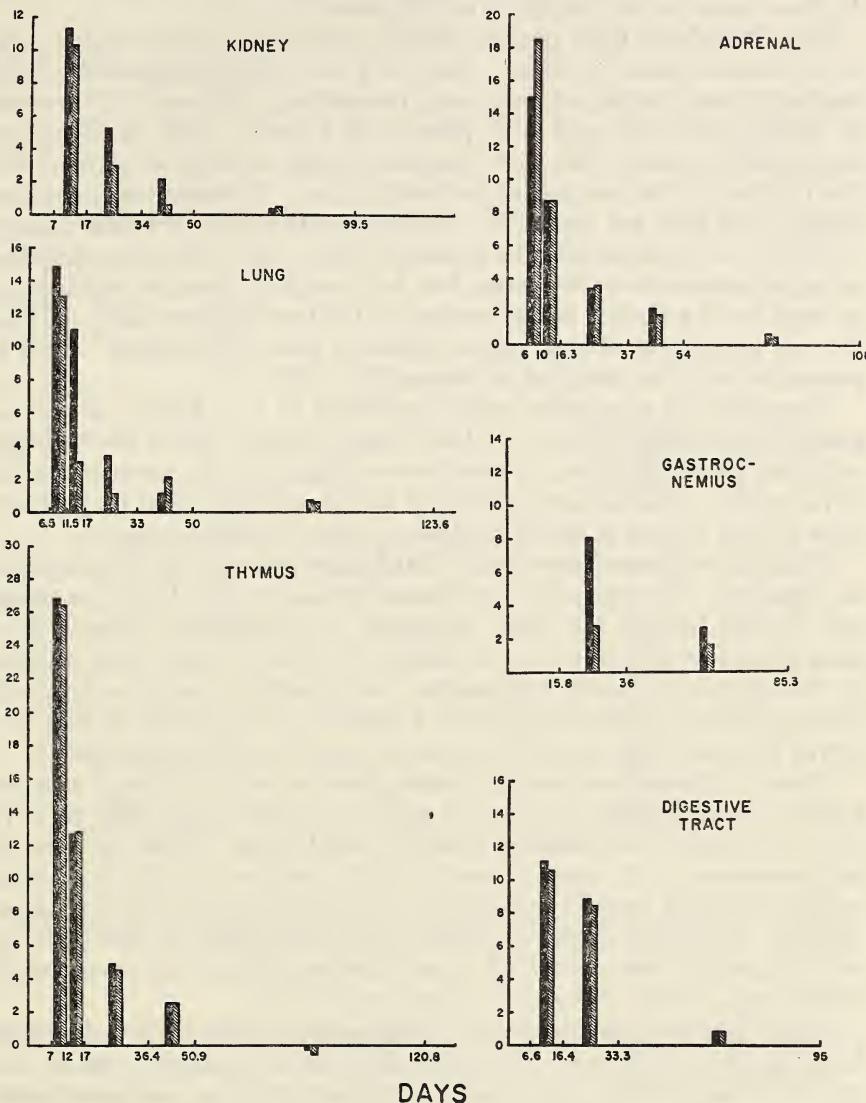


TEXT-FIGURE 5.—*Adrenal*. The curves for weight and number of nuclei rise with age (4).

The labeled nuclei of parenchymal cells were scattered throughout, not only in pancreas (figs. 1 and 4) and kidney (figs. 2 and 3), but also in many other organs, such as thyroid (fig. 5) and liver. It may be emphasized that even though cells undergoing mitosis temporarily lose some of their characteristic features, the cells labeled with H^3 -thymidine do not and therefore may be compared to other cells in the same population. When this comparison was carried out in the pancreas, kidney, liver, or thyroid, the labeled cells were quite similar to the unlabeled ones. Hence, the dividing cells of these organs were not "primitive" or "stem" cells, but were instead fully differentiated. This conclusion shows that, contrary to widespread belief (1), full differentiation of a cell is not necessarily associated with the loss of its ability to divide.

The *daily nuclear addition rate* of kidney and adrenal (text-fig. 6), as well as of pancreas, liver, and thyroid, was fairly high between 7 and 17 days of age in the rat, but decreased with age. During the 3d month of life (mean body weight, 240 g), the rate was only 0.45 percent for kidney and 0.23 percent for adrenal.

DAILY NUCLEAR ADDITION RATE (%)



TEXT-Figure 6.—Daily nuclear addition rate (gray columns) compared to the daily growth rate, as measured from weight changes (black columns). Both rates decrease with age considerably, but remain positive in all organs and tissues, except in thymus after the age of 50 days.

In the hope of finding out whether this addition of nuclei was accounted for by the mitoses present, the data were compared to *daily mitotic rates* obtained by Bertalanffy (11) in 240 g male rats. He measured these rates in glomeruli (0.37%), proximal convoluted tubules (0.31%), and distal convoluted tubules of kidney (0.42%). An average of the 3 figures

gave 0.37 percent for the mean daily mitotic rate of the kidney cortex. In the adrenal cortex the rate was 0.22 percent.

Even though the daily nuclear addition rate and the daily mitotic rate were obtained under conditions that were not strictly comparable, their similarity was striking, as they were, respectively, 0.45 and 0.37 percent in kidney, and 0.23 and 0.22 percent in adrenal. This similarity is interpreted to mean that there are just enough mitoses to account for the increase in the number of nuclei with age. Furthermore, since the mitotic rate does not exceed the nuclear addition rate, the cells arising in kidney and adrenal must be retained *in situ*—they must not emigrate or degenerate or otherwise become lost, but remain in the organ indefinitely as such (or, if a further mitosis occurs, as the two daughter cells). Thus the cell population of both organs increases with each mitosis. Such a population may be described as “expanding” (12).

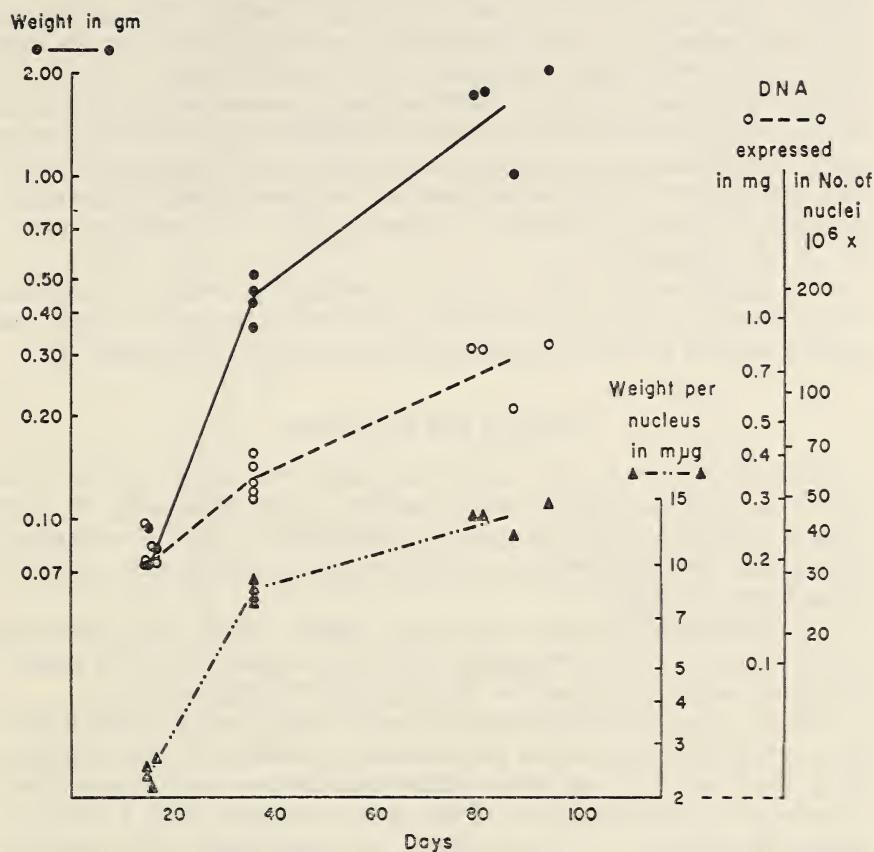
Expanding cell populations were then defined in 7- to 90-day-old rats as homogeneous groups of cells, in which scattered mitoses can be detected with colchicine or H^3 -thymidine, in numbers accounting for the increase in total DNA content. Under these conditions the life of each cell or its progeny lasts as long as that of the individual in whom the cell is present.

While the evidence demonstrates that kidney and adrenal are composed of expanding cell populations, preliminary data on the liver, pancreas, and thyroid lead to the same conclusion for these three organs. But data are not available to reach a definite decision for most other organs. In the absence of better information, we tentatively consider that cell groups showing scattered mitoses in a frequency comparable to that observed in kidney (figs. 2 and 3) consist of expanding cell populations.

Tissues.—Skeletal and cardiac muscle fibers are widely believed to have acquired their definitive number of nuclei at or soon after birth (2, 15); if so, they should be classified as static populations. However, Enesco and associates (4, 21) demonstrated that the number of nuclei in skeletal muscles increases twofold to fourfold between the ages of 16 and 90 days (text-fig. 7) and, furthermore, that muscle fiber nuclei of rats from 16 to 90 days of age may exhibit all stages of mitosis (22) as well as scattered labeled nuclei after H^3 -thymidine injection (12, 22).

In the tibialis anterior muscle, the daily nuclear addition rate calculated from the data of Enesco and Puddy (21) was 1.9 percent between the ages of 16 and 36 days. The daily mitotic rate was then estimated with colchicine in the same muscle, care being taken to record only those arrested metaphases which definitely appeared to be within the muscle fibers, and ignoring those figures regarded in this respect as possibles or probables. The results gave daily mitotic rates of 1.8 and 1.2 percent, respectively, in 16- and 36-day-old rats, *i.e.*, somewhat less than the daily nuclear addition rate. Thus, the DNA determinations showed an increase from 8.0 million muscle fiber nuclei at 16 days to 11.6 million at 36 days (21), whereas our daily mitotic rates are sufficient only to increase the number of nuclei from 8.0 to 10.7 million. Despite the discrepancy (attributed to the rigid method of counting arrested metaphases),

GASTROCNEMIUS



TEXT-FIGURE 7.—Weight and number of nuclei of gastrocnemius increase with age at a progressively decreasing rate. The weight per nucleus increases too, due to enlargement of muscle fibers (4).

we conclude that there are enough mitotic figures within muscle fibers to cause the increase in the number of nuclei with age. Skeletal muscle fibers would then constitute expanding populations.

Why does the mitotic activity of expanding cell populations gradually decrease with age? According to Rusch's model (1), when differentiation is completed the cell energy is diverted from duplication to specialized activities and the ability to divide is lost. However, it has already been pointed out that the cells of kidney (figs. 2 and 3), pancreas (fig. 4), thyroid (fig. 5) or muscle are fully differentiated and yet they can divide, though at a rate slowing with age.⁵ More impressive still is the possibility

⁵ It is possible that in fibers of skeletal muscle the nuclei undergoing mitosis are "satellite" nuclei located outside the fibers, though within the periodic acid-Schiff-stained membrane which encloses them (22). The dividing satellite cells might then not be differentiated cells.

of a rapid resumption of mitotic activity under the effect of adequate stimuli, *e.g.*, in liver cells after partial extirpation and in follicular cells of thyroid after injection of thyroid-stimulating hormone. An explanation proposed for the liver, which may apply to other organs, is that the cells release to the circulation a substance which inhibits their own growth. Partial extirpation of the liver would reduce the amount of circulating inhibitor, and the cells would then divide actively (23, 24). If so, the slowing down of mitotic activity with age would suggest that the concentration of the inhibitor in the circulation increases as the animals grow old (due either to increased secretion of inhibitor or more probably to its prolonged retention in the circulation).

After the 3d month of life, the rate of addition of nuclei to expanding cell populations continues to decrease, and toward the age of a year, the method used for DNA determination no longer detects any change (25).

Renewing Cell Populations

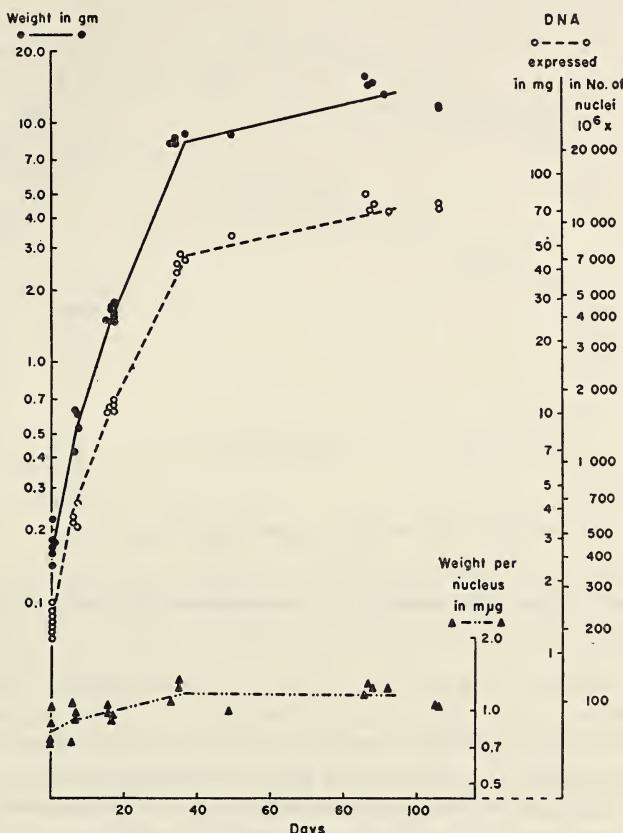
When the change in the number of nuclei with age is examined in structures such as digestive tract (text-fig. 8), lung (text-fig. 9), and thymus (text-fig. 10), the pattern is comparable to that of expanding populations, that is, there is an increase which is rapid at first and then slows down gradually.

The *daily nuclear addition rate* over a period during which the body weight was about 240 g was estimated to be 1.0 percent for the gastrointestinal tract, 0.47 percent for the lung, but 0 for the thymus.

And yet, when the *daily mitotic rate* was assessed, much higher figures were obtained: 68 percent for the intestinal epithelium of rats averaging 200 g [about half the nuclei of the wall of the intestine are in the epithelium, according to unpublished work of Enesco and Altmann (26)], 4.1 and 3.6 percent in the lung of 175 and 250 g rats, respectively (10), and 22.2 percent in the thymus of 200 g rats (27).

The large difference between the two rates indicated that, while many cells arose from mitosis in these structures, few were retained for addition to the cell population, or even none in the case of thymus. The discrepancy must be because many cells arising from mitosis are eventually lost by emigration, degeneration, or some other process. Indeed, it is known that the cells of the intestinal epithelium migrate from the crypts along the villus surface to the villus tips where they fall to their death in the lumen (7, 28), that the alveolar cells of the lung migrate out of the alveolar tissue to the alveolar spaces and from there to the bronchi and trachea, to fall into the esophagus (10), and finally that lymphocytes leave the thymus in large numbers to enter the blood circulation (29). The cell populations in which a high mitotic activity is thus balanced by a cell loss may be referred to as "renewing" and the organs containing such cells, as "renewal systems" (3, 12). *Renewing cell populations were then defined in 7- to 90-day-old rats as homogeneous groups of cells in which abundant mitoses can be detected with colchicine or H^3 -thymidine, in numbers exceeding*

DIGESTIVE TRACT

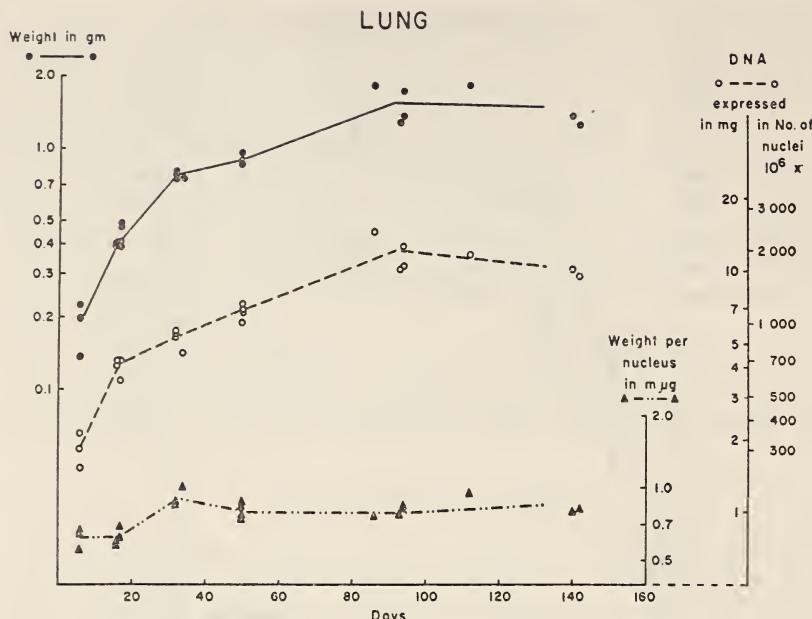


TEXT-FIGURE 8.—The curve for the number of nuclei in the digestive tract rises steeply until 34 days, but has a gentle slope thereafter. The changes in the curve for the weight per nucleus are not statistically significant (4).

those required by the increase in total DNA content, as the high cell production is largely balanced by a cell loss.

The magnitude of the cell production is high in certain renewal systems. Two examples will be cited, of which the first deals with a slowly renewing cell, the red blood cell. In man, 2×10^7 million red blood cells spend an average of 120 days in the blood stream. It is readily calculated that over 2 million cells must enter the circulation every second. Assuming that the mitoses of the precursor cells last an hour, then about 8 billion mitoses must be taking place at any moment in the human bone marrow for the maintenance of the stock of red blood cells (3).

The second example is provided by the epithelium of the small intestine of the rat. Unpublished data of Enesco and Altmann (26) in 90-day-old rats revealed that 1.38 billion cells per day are shed into the lumen of the small intestine alone. Considering the cells shed in pharynx, stomach,

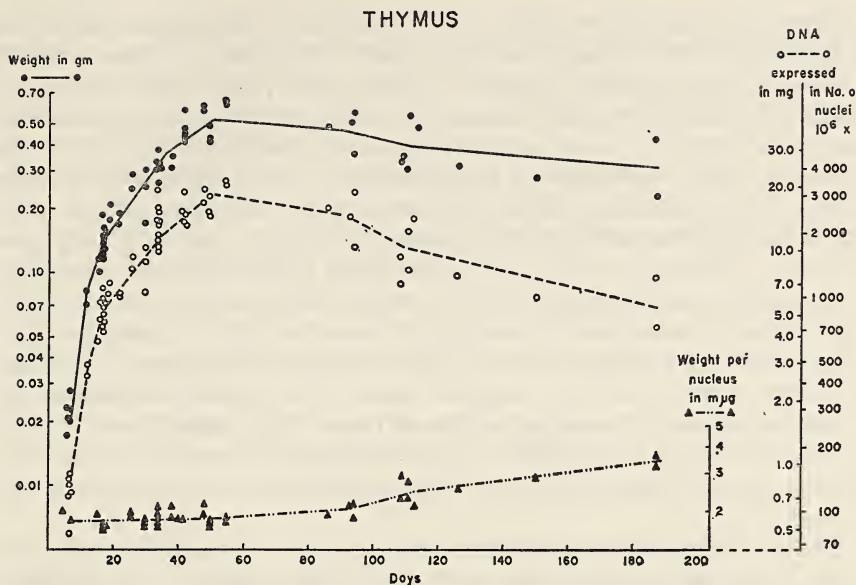


TEXT-FIGURE 9.—The pattern for lung is comparable to that of previously depicted organs (4).

and large intestine, it is likely that over 2 billion cells are released daily in the digestive tract. Since the total number of cells in the body of the 90-day-old rat is 67 billion (text-fig. 1), the daily loss would be about $\frac{1}{30}$ th of this number. It thus seems that the digestive tract produces and extrudes in about 30 days a number of cells equal to that of the whole body.

One may well wonder why nature has been so generous in providing an ever-renewing supply of cells for many populations. An explanation may be suggested for at least the protective epithelia, such as the epidermis and the lining of the digestive tract. While minor damage to these epithelia might elicit regenerative processes as in wound healing, the continuous supply of new cells insures that any damaged one is replaced automatically. The situation is not unlike that of a department store where electric light bulbs are replaced at fixed intervals whatever their condition is—a system that proved to be more efficient than waiting for the lights to burn out and having to be replaced one by one. Briefly, the constant influx of new cells anticipates damage and prevents the occurrence of weakened areas or gaps in the epithelium. Were such a renewal system to arise in the course of evolution, it would give the species a selective advantage and, therefore, would tend to become established as a hereditary characteristic (3).

Stem cells.—In a few epithelia, *e.g.*, that of the seminal vesicles, it seems that all the cells making up the epithelium have the ability to divide (12). Hence, as in expanding cell populations, though at a much more rapid rate, the differentiated cells of such epithelia may undergo mitosis. Differentiation would again be compatible with duplication.



TEXT-FIGURE 10.—The curve for the number of nuclei in the thymus rises steeply up to about 40 days, shows no significant changes between 40 and 90 days, and then goes slowly down. The thymus is the only organ in which the number of cells ever shows some decrease with time. The weight per nucleus curve remains level until 95 days and then rises (4).

In most renewal systems, however, division is chiefly seen in cells that are little or not differentiated, the stem cells. For instance, in the *stratified squamous epithelium of the esophagus* of the rat (30), the basal layer is composed of poorly differentiated cells which frequently undergo mitosis (fig. 6), whereas the cells of the spinous, granular, and keratinized layers never do. In fact, all cells of the basal layer have the ability to undergo mitosis, since in our 6-day experiment with frequently repeated H^3 -thymidine injections (18), they were all labeled (fig. 7). Hence, the cells of the basal layer are all stem cells.

While it has long been known that basal cells transform into spinous cells, which then become granular cells and keratinize, the use of H^3 -thymidine has demonstrated the irreversibility of this sequence. Indeed, once a cell has progressed ever so slightly beyond the stem cell stage (to become the so-called transitional cell), its differentiation goes on irrevocably (30). Hence, in the esophageal epithelium, duplication and differentiation are incompatible alternatives, as assumed classically (1). The crucial step in the life of its cells is when the stem cell is oriented either toward duplication or toward differentiation.

A similar situation is observed in the *seminiferous epithelium of testis*, where the stem cells are type A spermatogonia (31, 32). Again, once a cell goes beyond the stem cell stage, its evolution toward the spermatozoon will not stop.

Thus, mitoses of stem cells have to provide a continuous supply of differentiated cells, while maintaining their own stock. These mitoses must also serve another purpose. It has been shown above that the number of nuclei increases with age in many renewing cell populations (text-figs. 8 and 9) just as in expanding cell populations (text-figs. 3-7). However, while the increase is due to mitoses of fully differentiated cells in the case of expanding cell populations, it is due to mitoses of stem cells (or at least of cells with incomplete differentiation) in renewing cell populations.⁶ Furthermore, the number of nuclei in the latter increases less and less with age (text-figs. 8 and 9) and, when rats reach an advanced age—a year or more—no increase can be detected (33). In contrast, the cell replacement proceeds at a very rapid rate, even at that age. Quantitative data indicate that the turnover time of the jejunal epithelium (or rather an approximate estimate referred to as "total transit time") is 42, 52, and 54 hours, respectively, in mice aged 93, 372, and 940 days (34). Hence, there is little difference in the cell turnover of young and old animals.

Do stem cells divide differentially (text-fig. 11)? The significant feature of most renewal systems is that, even though few cells are retained, there is an intense cell turnover. The considerable cell production must, therefore, be approximately balanced by a considerable cell loss (3). The cell population is then said to be in "steady state" or at least, nearly so.

When single cells are considered, the steady state implies that for any cell lost from the population another cell has to divide and thus make up the loss. The simplest way for nature to achieve this aim would be for each division of a stem cell to produce one daughter cell that differentiates and another one that remains a stem cell. The implication would be that the dividing cell provides its two daughter cells with different amounts of material and thus leads them to a different fate. This type of division has been referred to as *differential* (or asymmetric or bivalent) (text-fig. 11). As a result of work on the production of spermatogonia, Rolshoven (35) proposed that in all renewal systems the division of stem cells is differential (2).

A search for differential mitoses in the *testis* revealed, however, that any two cells arising from the division of a type A spermatogonium were identical in monkey (32) and rat (36). Fifty percent of the pairs were composed of two cells oriented toward differentiation, while the other 50 percent consisted of paired cells remaining as stem cells. Hence, no evidence could be found that spermatogonia divide differentially.

Differential mitoses were also looked for in the *intestinal epithelium*. In comparing rats given the 6-day single or multiple injections of H^3 -thymidine, it could be shown that all crypt cells, except for a few at the bottom

⁶ The evolution of a differentiating cell may include a special type of mitosis which gives rise to daughter cells having a denser nucleus than the mother cell and a volume about half that of the mother cell (reductive mitosis). This is the case with the divisions of type B spermatogonia in rat testis yielding resting spermatocytes. The meiotic divisions giving rise to spermatids may also be included in this category. Reductive mitoses seem to occur in the course of the differentiation of lymphocytes in thymus (29) and perhaps also of bone marrow cells. Some of the reductive mitoses occur as in the central diagram of figure 11 (which depicts the cases in which a mitosis "transforms" a cell into two different daughter cells).

and those making up the upper third of the crypts, have the ability to divide, but any cell which migrates to the villus loses this ability (and may be looked upon as being differentiated). Hence, when a division occurs toward the bottom of the crypt, both daughter cells will divide again and again; but, when a division occurs just before the neck of the crypt is reached, the daughter cells are both likely to migrate to the villus and never divide again. Hence, the loss of the ability to divide and, presumably, the differentiation is not determined at the time of mitosis, but is dependent on the location of the cell.

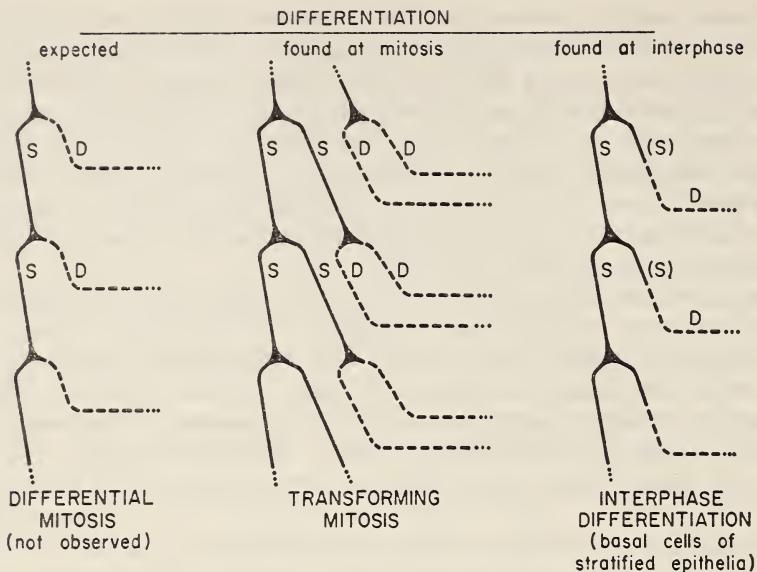
The search for differential mitoses in the *stratified squamous epithelium* of the esophagus (30) gave some insight into the problem. From what was said about steady state, it could be deduced that for every cell that moves out of the basal layer to become a spinous cell, *i.e.*, differentiates, there should usually be a cell added by mitosis to the basal layer.

If this result were achieved through a differential mitosis (35, 37), then each time a basal cell divides, one of the daughter cells would stay in the basal layer, while the other would move to the spinous layer. This possibility was examined by tracing the two daughters of basal cell mitoses. Cell maps were made from serial radioautographs of the esophagus at 24 and 48 hours after H^3 -thymidine injection (38) and paired daughter cells were identified by physical proximity and comparable grain counts. It was found that while a few pairs are composed of a basal and a spinous cell (in accord with the differential mitosis concept), more numerous pairs consist of two basal cells or of two spinous cells, in disagreement with the concept. It was concluded that either or both daughter cells of a mitosis may come out of the basal layer and they do so independently and at random (text-fig. 11, *right*). Hence, the cells do not divide differentially.

How is the number of cells maintained? The paradox is that the migration of a cell out of the basal layer is not the immediate consequence of the mitosis from which the cell comes. And yet, steady state requires that the number of cells leaving the basal layer be about equal to the number of mitoses. The problem was clarified by looking at mitotic figures. It is known that whenever a cell divides, its volume increases. Examination of the swollen dividing cells in the basal layer revealed that more often than not an adjacent resting cell was altered to a racket shape (fig. 7), as if it were being squeezed out by pressure from the dividing cell. More generally, the migration may be attributed to increase in population pressure in the basal layer. Whenever a cell divides, the pressure increases. And, on the average, for each cell added one must move out. Thus, the number of cells in the basal layer—the number of cells capable of division—would remain approximately the same (30).

It may be concluded that in the esophagus, as in the intestinal epithelium, the decision as to whether a stem cell will differentiate or continue dividing seems to depend on its environment.

Outlet for cells.—Much of what has been said above implies that an important feature of renewal systems is the existence of an outlet allowing



TEXT-FIGURE 11.—*Mode of division of stem cells in renewal systems.* Stem cells are depicted by solid lines (S). The mitoses of stem cells are indicated by a triangular thickening, from which the two daughter cells arise. In the diagram, the daughter cells may be other stem cells (S) or differentiating cells, indicated by broken lines (D). Diagram at left represents *differential mitoses*. No example of this type was found in testis, intestinal epithelium, and esophagus of the rat. It is tentatively concluded that differential mitoses do not occur in renewal systems. Center diagram is a schematic representation of *transforming mitoses*, *i.e.*, of mitoses giving rise to two daughter cell both different from the mother, though identical to each other. This type of mitosis has not been observed with certainty in stem cells. On the other hand, this type of mitosis may be seen in the course of differentiation. This is the case with type B spermatogonia yielding resting spermatocytes (21), with lymphocytes yielding smaller lymphocytes (29), etc. The daughter cells then have nuclei staining more darkly and of a volume about half that of the mother cell; hence the name of *reductive mitoses* proposed for them. Diagram at right depicts the only type of stem cell division which has been observed with certainty in stem cells of renewal systems (esophageal and intestinal epithelia). Briefly, the cells arising from stem cell mitoses would have similar potentialities. Under "local" or other influences, one or both or none of the daughter cells would differentiate during the interphase.

the eventual elimination of the cells produced. Thus, the differentiated cells arising in epithelia are cast off to the outside or into a lumen, the blood cells formed in hematopoietic organs pass into the circulation, and so on.

Comparison With Neoplastic Cell Populations

In embryonic populations, the numbers of cells appear to increase exponentially, first at a very rapid rate (over 100% per day) and later at a somewhat slower rate (about 60% per day). Toward the time of birth, exponential growth ceases and embryonic cell populations become static,

expanding, or renewing. Complete cessation of division characterizes *static* cell populations. Continuation of division, though at a progressively decreasing rate, occurs in *expanding* cell populations. In *renewing* cell populations, mitoses occur throughout life so as to insure a continuous replacement of the cells (text-fig. 12).

What happens when a cell population becomes neoplastic? Even though there is much information on the growth rate of tumors, the *daily mitotic rate* of neoplastic cell populations has only been measured by Bertalanffy, who found it to be constant at about 60 percent in the Walker carcinoma of the rat (text-fig. 13), 40 percent in a fibrosarcoma of the rat (39), and 12 percent in a mammary carcinoma of the mouse (40). The constancy of the rate was maintained as long as the tumors were solid and well vascularized.

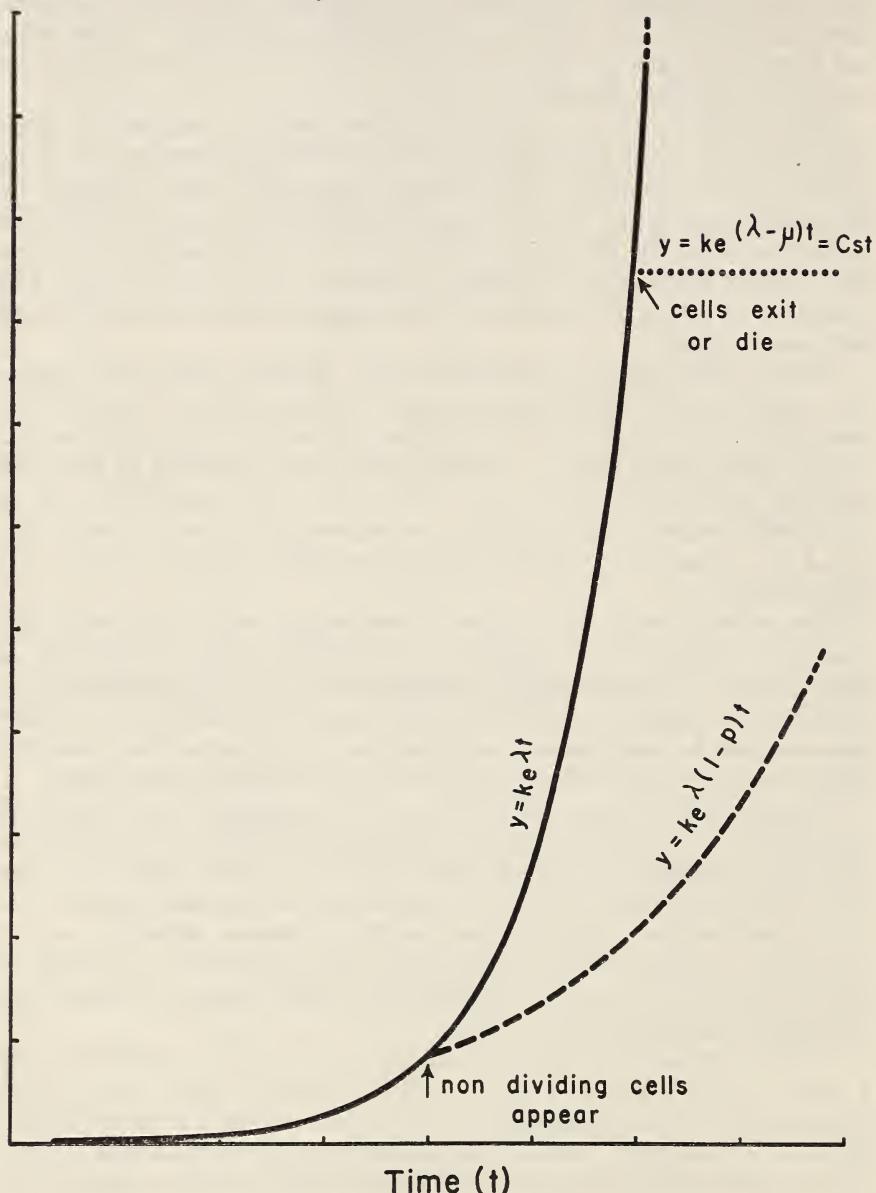
Precise information on the *daily nuclear addition rate* is also scarce. The cells of some Ehrlich ascites tumor strains (41-43) increased exponentially (text-fig. 14), with a doubling time of 0.75 day (daily nuclear addition rate, about 100%). In this case all cells seemed to be alive and dividing, at least during the first 5 days after inoculation (41). If, as Bertalanffy suggests, all cells were also retained in the tumors he investigated (40), these tumors would grow exponentially too (see also fig. 9 in reference 44).

Even when a small percentage of nondividing cells exists, as in the mammary carcinoma investigated by Mendelsohn (45), the increase in the number of cells should still be represented by an exponential curve, though a more slowly ascending one (*broken curve* in text-fig. 12). Briefly then, it may be emphasized, as others have (1, 2), that the continued ability to divide is an essential feature of neoplastic cell populations.

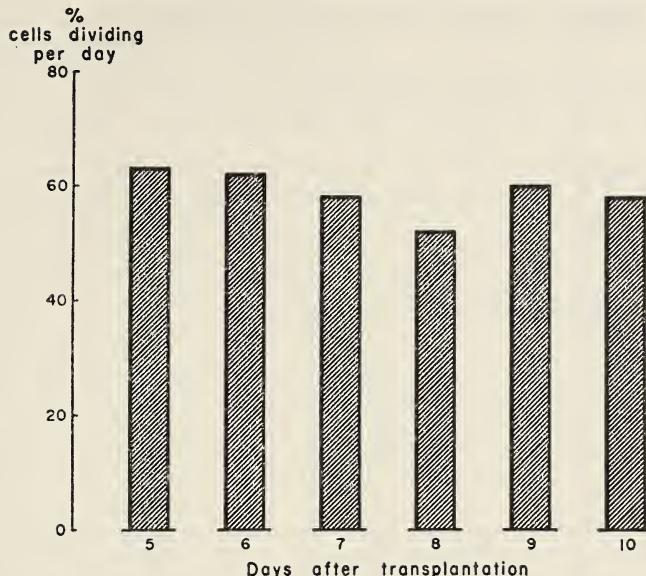
Let us briefly compare cell proliferation in neoplastic and normal cell populations. The cells of *expanding* populations produce daughter cells which, like neoplastic cells, have the ability to divide again; but they divide less and less frequently as the animals age, as shown by the decrease in the daily nuclear addition rate (text-fig. 6, kidney, adrenal), so that eventually the size of expanding cell populations tends to stabilize in contrast to the cells of tumors which keep on being added at the same rate indefinitely (text-figs. 13 and 14).

As with neoplastic cell populations, the *renewing* cell populations show a rapid, sustained rate of mitosis. Thus, the daily mitotic rate is about the same in the intestinal epithelium as in the Walker carcinoma (about 60%), even though this is one of the fastest growing tumors known. However, renewal systems give rise to cells which lose the ability to divide and eventually die, so that the increase in cell number is not exponential and, indeed, the size of the population tends to stabilize, just as in expanding populations. In contrast, all (40) or a high proportion (45) of the daughters of neoplastic cells retain the ability to divide, a fact which explains why even slowly growing tumors may be dangerous. Thus, Nakamura and Metcalf (46) described a lymphoma, the cells of which had a lower

Number of cells (y)



TEXT-FIGURE 12.—Comparison of the modes of increase in cell populations. *Solid curve* represents the size of a cell population that increases exponentially, *i.e.*, all cells have an equal probability to divide. (The relation of the number of cells, y , versus time, t , is given by the formula $y = ke^{\lambda t}$, where k is a constant and λ is the fraction of cells which divides by unit time.) If a certain proportion, p , of the cells does not divide, a more slowly ascending exponential results ($y = ke^{\lambda(1-p)t}$) which is depicted by the *broken curve* at *lower right*. If cells become lost or die (μ being the fraction of cells that are lost per unit time) and if they do so at a sufficient rate



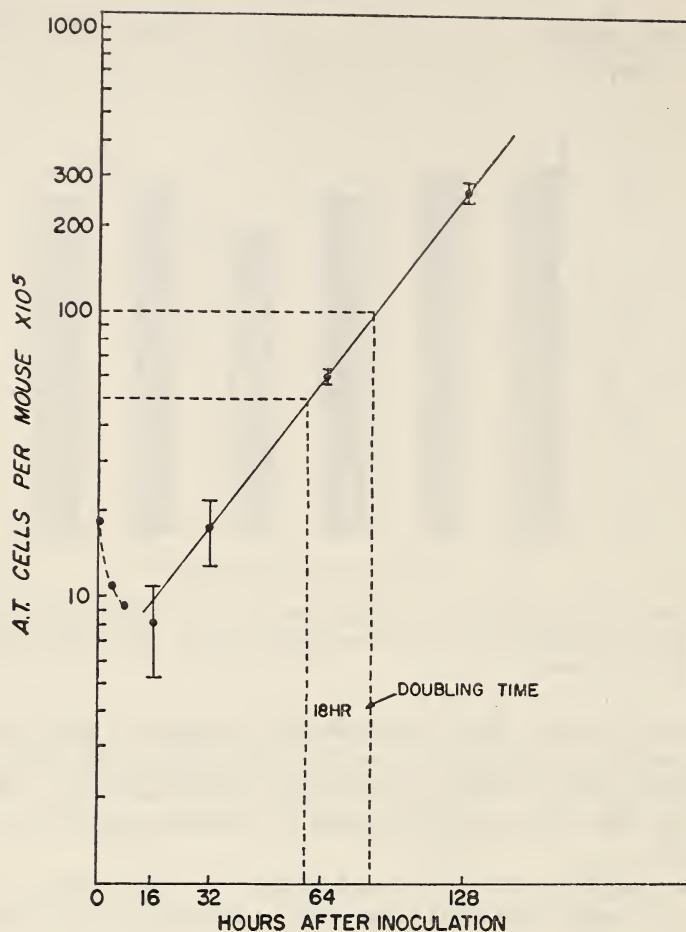
TEXT-FIGURE 13.—Daily mitotic rate of Walker carcinoma between 5 and 10 days after transplantation [diagram constructed from data in (39)]. The daily mitotic rate appears to be constant.

mitotic rate (1%) than those of thymus (3% according to these authors). However, while the thymus produces the nondivisible small lymphocytes, the lymphoma cells give rise to cells which continue to divide, and eventually they oust the normal cells from the thymus and choke off the circulation.

In some lymphomas, however, and perhaps in other tumors as well, there may be some turnover of the cells, a possibility which should be given careful consideration. Thus, a "remission" might be due to a balance being achieved between cell production and cell death, just as in renewal systems. However, if at any time cell production predominates, then the cells invade the circulation and behave as malignant cells.

Of course there are other differences between renewing and neoplastic cell populations. Renewing systems have an "outlet" allowing the escape of cells, whereas only few neoplasms do. Renewing cells do not have the

to balance the cell production, a steady state ensues. The *dotted line at upper right* represents the result [$y = ke^{(\lambda-\mu)t} = Cst$]. (From N. J. Nadler, unpublished data.) *Embryonic cell populations* increase exponentially at a rapid rate at first (*solid curve*) and more slowly later (*broken curve*). After birth, cell populations no longer increase exponentially. The *renewing cell populations* in steady state are depicted by *dotted line*. The same line may represent *static cell populations*, in which there is neither cell production ($\lambda = 0$) nor cell loss ($\mu = 0$). *Neoplastic cell populations* often increase exponentially (text-fig. 14) and may also be depicted by *solid curve*. If nondividing or necrotic cells appear, the population may then be represented by a more slowly ascending exponential, depicted by *broken curve*.



TEXT-FIGURE 14.—Growth curve of ascites tumor cells, obtained by plotting the log of the number of ascites tumor (A. T.) cells against time [from (41)]. Since the plot yields a straight line, the addition of cells occurs exponentially.

invasive properties of some neoplastic cells, etc. Yet the lack of mitosis-restraining influences in neoplastic cells seems to be the major difference between them and renewing cells.

RESUMEN

El aumento en el número de núcleos con la edad se ha estimado por el contenido de los órganos y tejidos de ratas jóvenes en crecimiento y expresado como la *tasa de adición nuclear diaria*. También se investigó las células en división, especialmente en radioautografías después de la inyección de timidina tritiada y después de la administración de colchicina. El uso de esta droga hizo posible medir la *tasa mitótica diaria*. Los resultados preliminares habían sugerido que las poblaciones celulares del embrión aumentan exponencialmente, pero hacia el tiempo de nacimiento el crecimiento

exponencial cesa. Las poblaciones celulares fueron entonces investigadas usando ratas que variaban en edad de 7 a 90 días.

En algunas poblaciones la actividad mitótica no es detectada y el contenido de DNA no cambia. Por lo tanto, el número de células permanece constante durante el período investigado. A estas poblaciones celulares se les refiere como *estáticas*, por ejemplo, las neuronas.

En otros órganos y tejidos, el contenido de DNA, y por consiguiente el número de células continúa en aumento, aunque a una tasa cada vez menor con la edad. Este aumento puede ocurrir en dos modos.

En el primer caso, las mitosis son aisladas y su número determina el aumento del contenido de DNA (y en consecuencia la adición de núcleos). Por lo tanto, todas las células añadidas por mitosis son retenidas. A estas poblaciones celulares se les refiere como *expansivas*, por ejemplo, las células parenquimales del hígado y del riñón, las fibras musculares, etc.

En el otro caso las mitosis son abundantes y su número excede enormemente del requerido para el aumento del contenido de DNA (y por lo tanto se producen muchas más células que las que son retenidas *in situ*). Las pérdidas celulares tienen lugar en tal modo que la elevada producción celular apenas excede las pérdidas. A estas poblaciones se les refiere como *renovantes*, por ejemplo, las células de la epidermis, el epitelio intestinal, el timo, etc.

En las poblaciones celulares neoplásicas la proliferación de las células y su progenie tiende a continuar indefinidamente. La producción celular excede grandemente la de las pérdidas celulares, aún en el caso de que algunas células cesen de dividirse y que tenga lugar cierto grado de renovación.

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PLATE 9

All figures are coated radioautographs of sections stained with hematoxylin and eosin; rats were killed after a single injection of H^3 -thymidine.

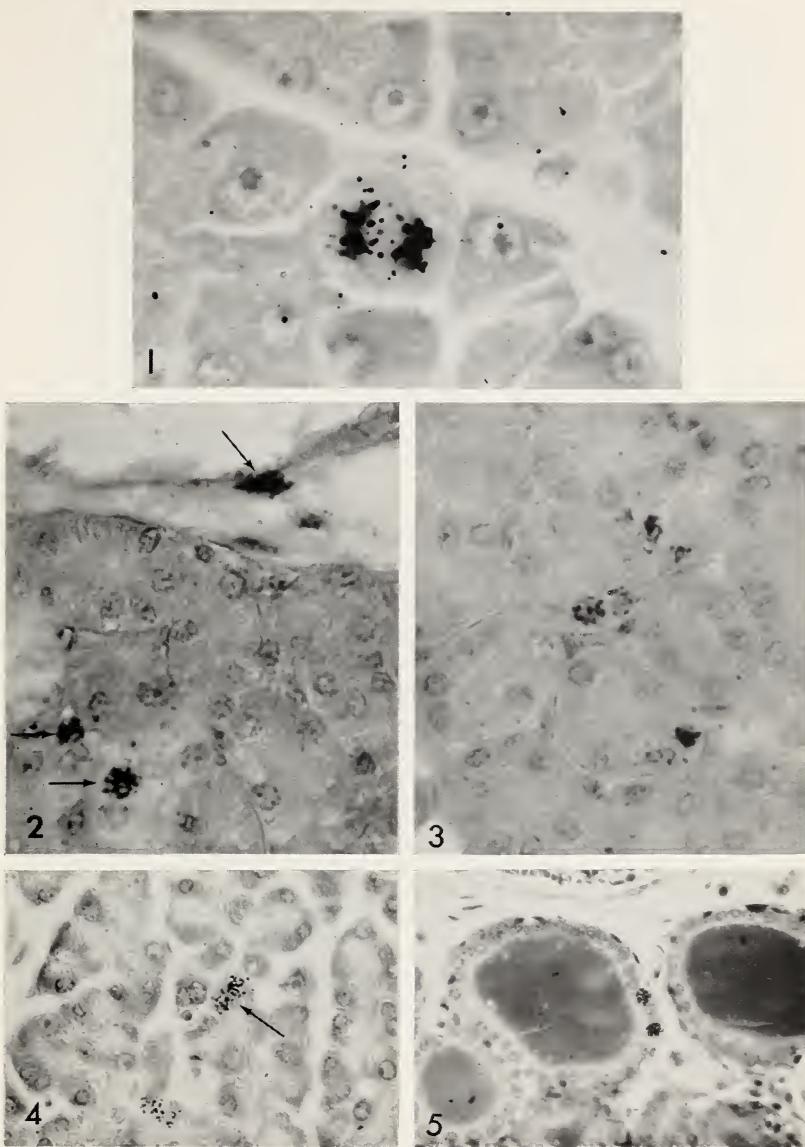
FIGURE 1.—Pancreas, 12 hours after injection, showing in *center* an acinar cell in anaphase with the two chromosome masses overlaid by silver grains. $\times 2,000$

FIGURE 2.—Kidney cortex, 8-hour interval. From the top down it is possible to see the kidney capsule with a dark spot which is a radioactive fat cell (*oblique arrow*) and the kidney cortex with two radioactive nuclei (*horizontal arrows*) in distal convoluted tubules. $\times 690$

FIGURE 3.—Kidney cortex, 24-hour interval. Scattered radioactive nuclei are seen in three proximal convoluted tubules. In each, careful examination reveals two radioactive nuclei side-by-side. (Each pair presumably arises from division of a labeled cell. That the two nuclei of any pair do not yield an equal reaction may be due to one of each pair lying deeper, and therefore, farther from the emulsion than the other.) $\times 690$

FIGURE 4.—Pancreas, 20-minute interval. Scattered labeled nuclei. A binucleate acinar cell with both nuclei showing a reaction is present in the center of the field (*arrow*). $\times 515$

FIGURE 5.—Thyroid gland, 1-hour interval. At *center right*, two follicular cells lining a large follicle show a reaction.



LEBLOND

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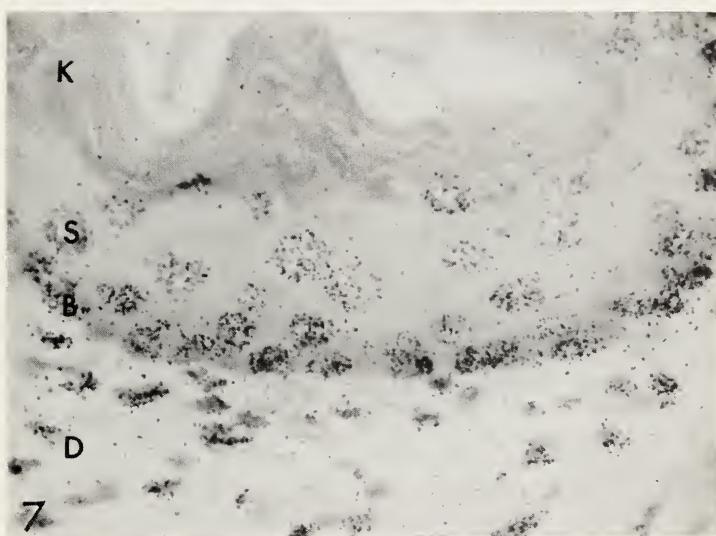
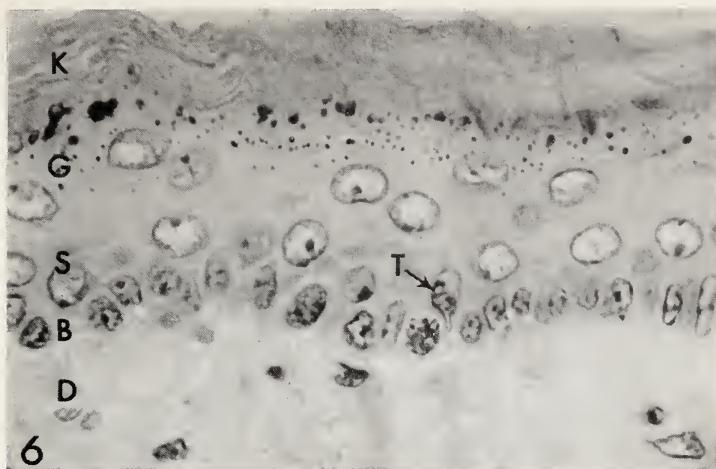
PLATE 10

FIGURE 6.—Esophageal epithelium of 200 ± 10 g male rat. Bouin fixation. Hematoxylin and eosin stain. $\times 875$

From the base up, *note* the dermis (*D*), the basal layer with crowded cells and nuclei usually perpendicular to surface (*B*), the spinous layer with fairly large cells, light nuclei and prominent nucleoli (*S*), the granular layer (*G*) and keratinized layers (*K*). At *T*, there is a cell considered to be transitional between basal and spinous cells. This cell seems to be squeezed out of the basal layer due to pressure from the prophase at its left.

FIGURE 7.—Coated radioautograph of the esophageal epithelium of a 16 g rat given $0.1 \mu\text{c}$ of H^3 -thymidine per g body weight every 3 hours over a 6-day period and killed 1 hour after the last injection (18). $\times 600$

All nuclei in basal, spinous, and granular layer are labeled. (The label is lost as the cells are keratinized. Many, but not all, nuclei of the dermis are also labeled.)



DISCUSSION

We will now try to answer the question about cell renewal systems and how they differ from cancer.

Leblond: For some cell populations, *e.g.*, seminal vesicles, the only safeguard that prevents the cells from developing to excess, as cancerous cell populations seem to do, is the fact that they push each other into the lumen.

Hodgson: Dr. Leblond, for the kidney in which there is an expanding population, do the number of nephrons change from the 7-day-old rat to the adult or do the number of cells per nephron only change?

Leblond: Only the number of cells per nephron, as shown in unpublished work of M. Enesco.

MacCardle: * I have two questions. Did I understand correctly that you think all the basal cells of the epidermis divide by mitosis? Do the squamous cells divide? I wonder whether you studied psoriasis, in which the epidermis proliferates (acanthosis) with great speed but forms no tumor. In psoriasis, you would not be concerned so much with necrosis as in the tumor. Dr. Van Scott has been interested in the turnover rate in psoriasis. It looks as though it requires only about 4 days to replace the whole psoriatic epidermis, compared with 27 days for normal epidermis. Also it was found by making models from horizontal sections of epidermis, that the mitotic figures were confined to the basal cell layer, those appearing to be in the spinous layer being really at the edge of a papilla which may appear in the next histological section.

Leblond: While I cannot add to your interesting comments about psoriasis, it can be shown that the small, dark cells of the epidermis, referred to as basal cells (and making up the 1-3 basal layers) all have the ability to divide. This can be shown by continuous injections of thymidine. In the esophageal epithelium which was discussed in the paper, mitoses are restricted to the basal layer.

Cristoffanini: * A daily mitotic rhythm has been shown in young liver by J. Echave-Llanos, from Instituto de Patología General y Experimental, Facultad de Ciencias, Universidad Nacional de Cuyo, Mendoza, Argentina. I wonder how much mitotic rhythm would influence this type of population because (at moment of timing) your figures are quite good. My second point is—I wonder if you have looked into the problems in chronic myelocytic or chronic lymphocytic leukemia, where in one you cannot find so many mitoses, and in the other almost none. Both come to a stable period; in the first one they keep multiplying and you see the mitoses, in the other one you do not see them; it may be assumed that they have a different rate of elimination. There is also an increased rate of destruction.

Leblond: While there is a diurnal variation in the mitotic activity of liver, kidney, and other organs, the colchicine mitoses at 4 or 6 different intervals over a 24-hour period were added to give the "daily mitotic rate." Hence, our figures take into account any diurnal variation. As for the problem of cell turnover in various types of leukemias, it is an important one requiring further investigation.

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Stem Cells of the Hematopoietic and Lymphatic Tissues¹

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SUMMARY

The properties of hematopoietic and lymphatic tissue stem cells, which we assume are acted on by carcinogenic agents to give rise to the many tumors of blood-forming organs, were studied. Hematopoietic cells from normal or pretreated donors were transplanted into heavily irradiated mice to investigate the origin, distribution, and functional capacity of stem cells. The presence and proliferation of transplanted cells in chimeras were assessed by survival data, histology, uptake of Fe⁵⁹ by erythropoietic elements, and serological identification of donor-type cells. Recent experiments have shown that hematopoietic and lymphatic tissue stem cells, even in the adult mammal, are not confined to the well-organized hematopoietic and lymphatic organs (bone marrow, lymph nodes, and spleen), but can be found in peripheral blood and among the free cells of peritoneal fluid. Peritoneal fluid cells from

chimeras also are of donor type. This indicates that the macrophages, mast cells, and other types ordinarily found in the peritoneal cavity originate in the bone marrow. Because stem cells are circulating and may be found in the tissues and body cavities, they may be exposed readily to carcinogenic agents. It is even possible that a carcinogen, deposited subcutaneously or intramuscularly, may come in contact with circulating stem cells and produce a local tumor from them, rather than from the adjacent connective tissues. For the *in vitro* study of the action of external agents on stem cells, it would be helpful to isolate them, possibly by mechanical or physiological techniques mentioned in this paper. Transplantation of stem cells from different topographical sites after their *in vitro* exposure to carcinogens might be of considerable interest in the induction of cancer.—Nat Cancer Inst Monogr 14: 151-168, 1964.

BASIC STUDIES on carcinogenesis naturally center on cells of the adult mammal that are capable of division, for these are the stem cells which give rise to benign and malignant tumors of any particular organ. Stem cells, although sometimes identifiable morphologically in tissue sections, cannot easily be obtained from many organ systems for *in vitro* or other studies.

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

One method long in use in the study of dividing cells is the observation in fixed tissue preparations of cells undergoing mitosis. If one counts the number of divisions per given number of cells, a mitotic index can be derived. By the use of colchicine, an agent that arrests cells in mitosis at metaphase, information can be gained about turnover times.

Another favorite tool in such studies is H^3 -thymidine which labels dividing cells and permits one to follow the daughter cells through a few subsequent divisions. Labeled DNA precursors in general have been very helpful, not only in the search for but also in the characterization of dividing cells. Leblond and his associates (1) used such labeling techniques to good advantage in studying cell renewal patterns.

Nossal and Mäkela (2), Gowans (3), Porter and Cooper (4), and others currently engaged in immunological studies have also used DNA labeling to follow cells of morphologically recognizable types during an immune response. Their data have led to the well-known disagreement on the role of the small lymphocyte in the immune response. Porter and Cooper (4) and Gowans (3) have separately advanced the view, based on behavior of labeled thoracic duct lymphocytes after injection into homologous newborn rats, that the small lymphocyte transforms, without division, into a larger, homograft-reactive cell in the host's lymphatic tissues. Carstairs' (5) observations on short-term cultures of human blood tend to support the small lymphocyte transformation idea, for larger, new forms appeared as small lymphocytes disappeared in the absence of any change in total cell count and before the onset of cell division.

However, Nossal and Mäkela's study (2) of the rat's popliteal lymph node during the course of a secondary response to *Salmonella* flagella argues strongly against the small lymphocyte as an immunologically active cell. In this case, a single injection of H^3 -thymidine was given 2 hours before the secondary antigen injection, so that only cells dividing prior to stimulation were labeled. Following the cells of the node at intervals after antigen, and including material from appropriate controls, these workers found that the large lymphocytes or germinal center cells—but not small lymphocytes—were heavily labeled at first. Germinal center cells were labeled throughout the observation period, and gave rise to all plasmablasts and plasmacytes appearing within the next few days. Essentially no unlabeled plasma cells were found. Small lymphocytes became labeled during the experiment, and by the 4th day 15 times as many were labeled as in controls.

The actual origin or renewal mechanism for the germinal center cell cannot be demonstrated by the data presently available (6). If one discounts the small lymphocyte for the moment, local reticular cells seem a logical choice. However, Nossal and Mäkela (2) found that they too were essentially unlabeled in their experiment.

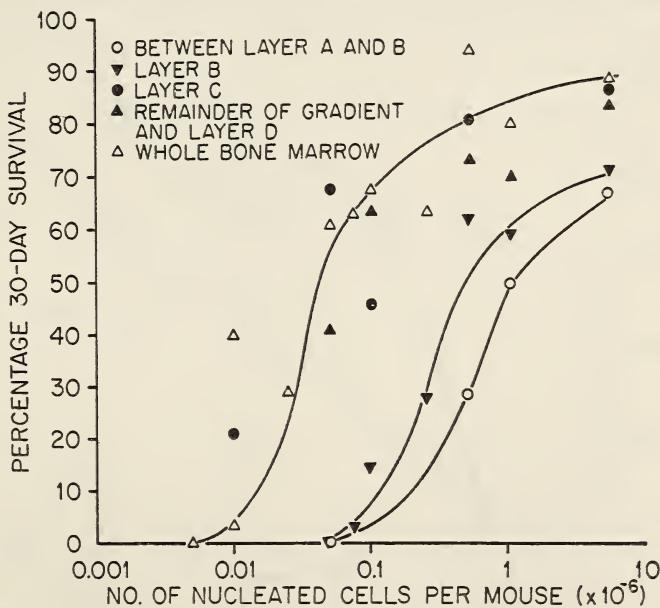
Yoffey (7), perhaps the best known proponent of the small lymphocyte as stem cell, proposes that it is capable of differentiating not only into immune cells of all categories but into granulocytes and erythrocytes as well. He has adduced many experimental data, including those from his

own studies, in support of this hypothesis. The evidence, although highly suggestive, remains circumstantial, and a conclusive demonstration of a pluripotential capacity of small lymphocytes has not been accomplished.

Our own work on stem cells of the hematopoietic and lymphatic tissues has been concerned with mechanical and physiological cell separation (8, 9), the location or topographical distribution of stem cells in the adult mouse, and with the capacity of stem cells to differentiate after transplantation to a heavily irradiated recipient (10, 11).

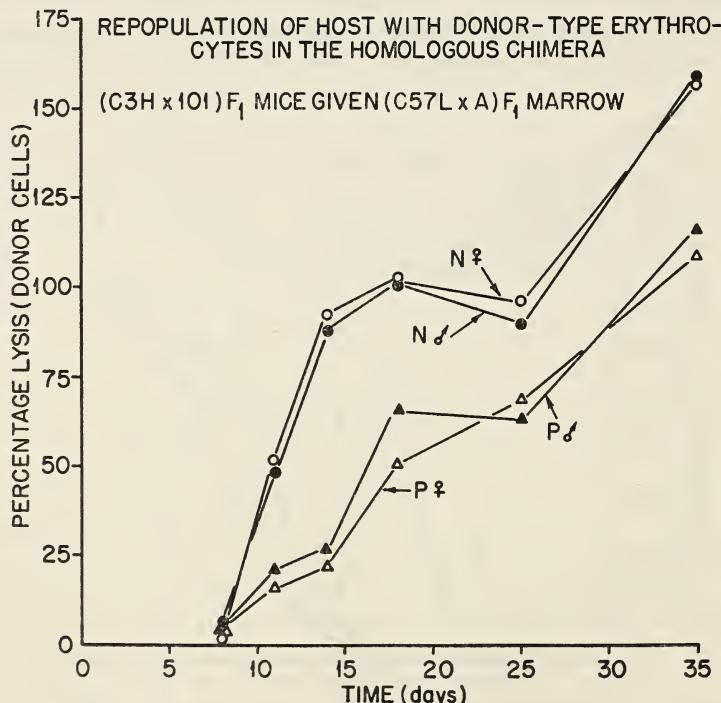
Physical approaches to the separation of bone marrow cells into pure suspensions of individual cell types, to date, have been less than a complete success. Yet, the usefulness of a good physical separation method is obvious and warrants continued investigation. In our laboratory some degree of separation was achieved by density gradient centrifugation in bovine serum albumin (BSA). However, the mononuclear cells separated (fig. 1) were less effective than whole bone marrow when tested for ability to promote survival of heavily irradiated recipient mice (text-fig. 1).

Several investigators, including Hilal *et al.* (12) and Mel (13), are currently studying bone marrow cell separation and have reported success in separating out particular cell types; their preparations were evaluated morphologically. Mel's use of stable-flow free boundary sedimentation appears to be a good method, and we can look forward to experimental results from testing the function of the elements separated from marrow.



TEXT-FIGURE 1.—Survival of lethally irradiated mice injected with various dosages of separated isologous bone marrow cells. Both male and female (C57BL \times 101) F₁ mice were used (8).

Another approach to the characterization of hematopoietic stem cells is physiological separation or, more exactly, the shifting of cell populations by physiological manipulation. The particular method we used (9) was artificial polycythemia to produce a white, or nonerythropoietic, marrow. This plethoric marrow (PBM) was compared in transplantation studies to normal, untreated marrow (NBM) with respect to its ability to support erythrocyte repopulation of irradiated recipients. The results showed that red cells from the plethoric marrow transplant were first detectable with serological techniques at the same time (8 days) they could be found in chimeras that had received normal marrow (text-fig. 2). Both types of marrow gave rise to permanent transplants that supplied all circulating erythrocytes. One striking difference between PBM and NBM chimeras was that the former group showed only a slight, transient anemia, whereas the latter—NBM chimeras—showed severe lowering of the hematocrit with slow recovery and relatively poor 30-day survival. It was postulated that platelet depression and consequent hemorrhage were largely responsible for the severe anemia, and recent preliminary studies show that much better platelet recovery is seen in recipients of PBM than of NBM. Many interesting experiments remain to be done on the stem cell pools existing in polycythemia and other abnormal marrow situations.



TEXT-FIGURE 2.—Percentage hemolysis with donor-specific reagent compared to normal mice of donor type taken as 100 percent. Eighteen mice in each normal (N) and 27 in each plethoric (P) group. Note that donor cells are detected at the same interval initially in N and P groups (9).

Work on the location or topographical distribution of stem cells involved a number of experimental procedures, most important of which were techniques permitting identification of donor cells in various organs of the host animal. Antigens present on donor-derived cells but absent from host cells were the markers used. To avoid graft-versus-host immune reactions that might ruin the experiment, donor cells from F₁ hybrid mice were often used, and one of the parental strains represented in the donor was the irradiated recipient. Several of the specific serological techniques have been used extensively in tissue transplantation work to demonstrate chimerism. The methods include hemolysis and agglutination of erythrocytes (14, 15) and death, as measured by dye uptake, of nucleated cells by cytotoxic action of specific, isoimmune sera (16). Another method used to gauge the success of transplantation was the measurement of Fe⁵⁹-uptake by the spleen and red cells of an irradiated recipient (table 1). Fe⁵⁹-containing erythrocytes could then be reacted with specific hemolytic serum and the lysate counted for Fe⁵⁹ activity (table 2). Very young red cells, those labeled within 24 hours after Fe⁵⁹ injection, are extremely susceptible to immune lysis. This refinement of the hemolytic technique, worked out with Hodgson in our laboratory (10), can be very useful in assessing erythropoiesis early after transplantation.

In addition to iron uptake and serological identification of donor-type cells in the recipient, standard observations on survival, and gross and microscopic examination of chimeric tissues helped to evaluate differentiation of transplanted cells.

Lymph node cells were tried as a source of hematopoietic cells in F₁ into irradiated parent (P) transplantations, but survival of recipients was never seen. Good regeneration of lymph nodes and spleen white pulp

TABLE 1.—Fe⁵⁹ incorporation by individual chimeric mice

Irradiated host*	Leukocyte donor	Leukocyte dose (millions)	Days after leukocyte injection	Hours after Fe ⁵⁹ injection	Percentage of injected iron dose† found in:	
					Spleen	Peripheral erythrocytes
B10 . D2	B6D2F ₁	20	11	18	{ 10.70	13.30
		None			0.18	0.03
		20	12	42	{ 5.70	18.10
		None			0.29	0.01
		40	9	22	{ 2.80	6.50
		None			5.40	4.60
D2	B6D2F ₁	25		22	2.90	4.40
		None	8	24	0.15	0.01
		None			{ 5.40	2.30
		50	13	39	0.40	0.80
C3	BC3F ₁	75	13	39	{ 2.1	12.1
		54			—	24.6
		46	11	24	—	13.1
		36			—	29.5
		54			—	5.1
		46			—	9.4
		36			—	13.9

*Each recipient except C3 was injected with ESF 48 hours before Fe⁵⁹ injection.†As iron citrate, 0.5 μ c; specific activity 10 μ c per μ g.

TABLE 2.—Determination of strain type of Fe^{59} -labeled erythrocytes from chimeras

Host	Strain	Donor		Tested (days after cellular treatment)	Percentage of specific hemolysis* in immune serum, determined by Fe^{59} count	
		Cell type and dose (millions)			D2 anti B6	B6 anti D2
D2	B6D2F ₁	Leukocytes	25	9	47.3	39.8
			50	13	77.1	74.8
			75	13	78.5 63.0 73.9	70.0 50.0 88.0
D2(3)	D2	Bone	0.2	9	0	>100†
D2(3)	B6D2F ₁	marrow cells	0.2	9	93.0	76.3
B6D2F ₁ (3)	B6D2F ₁		0.2	9	79.0	61.3
D2	B6D2F ₁	Leukocytes	50 75	56	32.2‡ 23.8‡	74.6‡ 66.0‡

*Values for individual mice except for bone marrow cells from chimeras where average values for 3 mice are given.

†See (10) for explanation.

‡Antisera of same specificities but of different immunizations from those used in rest of table.

was evident within the first 2 weeks after treatment, and many large antibody-forming cells and mature plasma cells could be seen (fig. 2). This was not surprising in view of much previous work showing that lymph node cells, transplanted to another animal, can take part in serum antibody production as well as homograft reactions (17-19). However, no stem cells were present that could give rise to red cell formation, judged from inability of a total of 33 mice in 5 experiments (given 0.5 to 2×10^8 cells) to incorporate any appreciable amount of Fe^{59} in the subsequent 7- to 11-day period. Histologic examination occasionally revealed small foci of erythropoiesis, but in general confirmed the lack of myelopoiesis. Megakaryocytes were occasionally found, but with no greater frequency than in heavily irradiated, untreated mice. The lack of hematopoietic activity of lymph node cells found in our experiments is in agreement with similar findings of Micklem and Ford (20). It is, perhaps, too early to correlate lymph node cell transplantation results with attempts to derive hematopoiesis from thoracic duct lymphocytes because results with thoracic duct cells are conflicting (21, 22), and reports of their failure to repopulate marrow may represent inadequate attempts.

From lymph node cell transplantation studies, it would appear that the presumably multipotential, local reticular cells in nodes either appear in these suspensions in numbers too small to be detected by our methods, or they are not truly multipotential. Yet we know from bone marrow transplantation studies that stem cells capable of repopulating all lymphatic tissues of the mouse are present in marrow.

Because of many indications in the literature that hematopoietic stem cells might be present normally in peripheral blood [definite evidence having been published that lymphopoietic stem cells circulate (17, 19)], peripheral leukocytes from F₁ hybrids were transplanted into irradiated strain P mice. After failures involving many mice, more than 20 chi-

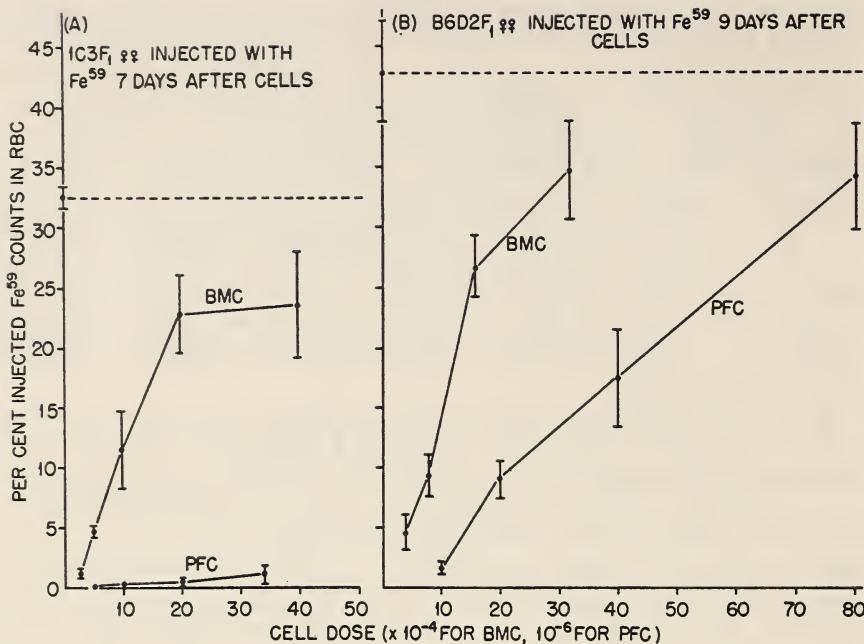
meras of several nonisologous combinations were eventually obtained, some of which survived and retained both hematopoietic and lymphopoietic transplants well beyond 1 year. By the criteria outlined, it was determined in many cases within the first 2 weeks after transplantation that peripheral leukocytes were differentiating into erythrocytes and granulocytes as well as lymphocytes. Cell dose requirements were not thoroughly explored, but survival has been recorded after as few as 5×10^6 isologous leukocytes. Thus, it was clearly shown for the mouse that hematopoietic stem cells normally circulate in the blood. Similar data pertaining to dogs (23) and guinea pig (24) have been published, and Freireich and Levin (25), at the National Cancer Institute, National Institutes of Health, Bethesda, Maryland, are currently investigating this problem in man.

From blood leukocytes a logical next step was to look for hematopoietic activity among free cells of the peritoneal fluid (11). Again, F_1 into irradiated P transplantation was used because of the high proportion of immunologically competent cells known to be in the peritoneal fluid. Cells were collected from many donors for a single experiment, the yield from a single donor being approximately 5×10^6 cells, and suspensions were adjusted so that the required dosage (in the range from 10^7 to 10^8) could be given intravenously in a volume of 1 ml. Cells either from untreated "normal" mice or from mice stimulated with glycogen were transplanted. In 14 of 16 experiments, there was definite evidence of donor-type hematopoiesis in the irradiated hosts. One of the most convincing criteria of successful transplantation was long-term survival with serological evidence of red and white blood cell chimerism.

To compare the hematopoietic capacity of peritoneal fluid cells to that of bone marrow cells, Fe^{59} -uptake was studied in lethally irradiated recipients given several dosages of isologous cells from either marrow or peritoneal fluid (text-fig. 3). The results indicate that a given inoculum of bone marrow contains around 250 times more stem cells than a comparable dose of peritoneal fluid cells.

The origin of peritoneal fluid cells, now known to have hematopoietic capacity, has long interested histologists and immunologists. At the beginning of this century Beattie (26) published experimental results bearing on the origin of these cells in unstimulated or stimulated guinea pigs and rabbits and in acute peritonitis in humans. He studied not only the free cells but also the organized tissues of the omentum and peritoneum.

Others (27, 28) since Beattie have made experimental studies in various species, and in general have concluded that the free cells are derived partly from endothelium of the serous membranes, blood vessels, lymph vessels, and lymph sinuses, partly from mononucleated leukocytes, and partly from lymphoid tissue surrounding the vessels. The autochthonous versus the hematogenic origin is a very old argument, and in addition to the many-sites conclusion just stated, there have been proponents of both extreme points of view. As early as 1905 Helly (29) stated that "exu-



TEXT-FIGURE 3.—Twenty-four-hour erythrocyte Fe^{59} -uptake as a function of peritoneal fluid cell or bone marrow cell dose. Standard errors shown (11). (A) $1\text{C}3\text{F}_1$ females injected with Fe^{59} ($0.5 \mu\text{c}/\text{mouse}$) 7 days after injection of cells; (B) $\text{B}6\text{D}2\text{F}_1$ females injected with Fe^{59} ($0.5 \mu\text{c}/\text{mouse}$) 9 days after injection of cells.

dative cells are derived from blood cells, and those in turn from the more primitive cells of the hematopoietic tissues."

The radiation chimera gave us an opportunity to look at the question of origin because the chimera's bone marrow, lymph nodes, and blood cells are of donor type, whereas serosal lining cells and submesothelial connective tissue cells are presumably of host type (30). Identification was made of antigens present on cells taken from the peritoneal cavities of chimeras that retained hematopoietic transplants. That they retained transplants was determined in all cases by usual serological testing of circulating red and white cells or by cytotoxic typing of marrow and lymph node cells at the time of peritoneal cell analysis. The chimeras tested were long term, *i.e.*, tested several months after X rays and treatment, and they had been transplanted with several kinds of nonisologous cells including bone marrow, fetal liver, blood leukocytes, and peritoneal fluid cells from mice, and bone marrow from rats. In every case tested that retained a hematopoietic graft, the peritoneal fluid cells were of donor type. This was true regardless of the tissue origin of the grafts and whether or not an irritant had been used to produce an exudate.

These results, supported by those of Balner (31) who studied macrophages within the first 2 months after homologous bone marrow transplantation, can only be interpreted to mean that all free cells of the peri-

toneal fluid, including mast cells, are derived from hematopoietic tissue. The alternative explanation—that submesothelial connective tissue cells contribute—requires that such tissue histiocytes be truly “resting wandering cells,” as Maximow (32) called them, and that they be renewed in the adult mammal from stem cells of the marrow and not from a self-sustaining local pool.

The extent to which local lymphatic tissues might contribute to free peritoneal fluid cells is not clear, as the cell type of these tissues in radiation chimeras is always of donor origin.

The additional possibility exists that although this renewal system obtains for the chimera, the normal, unirradiated mouse draws his cells, at least in part, from serosal lining cells and from reticular cells that belong to a locally self-sustaining, primitive pool. This possibility seems most unlikely in view of the normal appearance of peritoneal fluid from the chimera with respect to cell count and cell type, both before and after stimulation with such agents as India ink, endotoxin, and glycogen.

CONCLUSION

The research work that I have described is part of our program on experimental hematatology and is directed mainly toward providing basic information for the problem of tissue transplantation as a treatment after lethal whole-body radiation injury.

In the context of the present Conference one might point out that leukemogenic agents and situations must be acting on the stem cells of the hematopoietic and lymphatic tissues. Because stem cells are circulating and may be found in the tissues and body cavities, opportunity for exposure to these agents may be considerable. It is also possible that a carcinogen deposited subcutaneously or intramuscularly, even in a very insoluble vehicle, may come in contact with circulating stem cells and produce a local tumor from them, rather than from the adjacent connective tissues.

Furthermore, if we want to study the action of these external agents on stem cells *in vitro*, it would be helpful to isolate them, perhaps by the mechanical or physiological techniques mentioned in the present work. Transplantation of stem cells from different topographical sites after their *in vitro* exposure to carcinogens might be of considerable interest in the induction of cancer.

RESUMEN

Se han estudiado las propiedades de las células stem de los tejidos hematopoyéticos y linfáticos. Asumimos que sobre dichas células actúan los agentes carcinógenos para inducir los muchos tumores de los órganos formadores de la sangre.

Las células hematopoyéticas de los dadores normales ó previamente tratados, fueron transplantados a ratones intensamente irradiados, con el objeto de investigar el origen,

la distribución y la capacidad funcional de las células stem. La presencia y proliferación de las células transplantadas en las "quimeras" fueron determinadas por 1) datos de sobrevida, 2) histología, 3) captación de Fe⁵⁰ por los elementos eritropoyéticos, y 4) identificación serológica de las células del donante.

Experimentos recientes han mostrado que las células stem de los tejidos hematopoyéticos y linfáticos, aún en los mamíferos adultos, no están limitadas a los órganos hematopoyéticos y linfáticos bien organizados (médula ósea, nódulos linfoides y bazo), sino que pueden encontrarse en la sangre periférica y entre las células libres del fluido peritoneal. Las células de tal fluido de las "quimeras" son también del tipo del dador. Esto indica que los macrófagos, mastocitos y otros tipos celulares que se encuentran ordinariamente en la cavidad peritoneal se originan en la médula ósea.

Como las células stem están circulando y pueden encontrarse en los tejidos y cavidades orgánicas, éllas pueden estar fácilmente expuestas a los agentes carcinógenos. Es aún posible que un carcinógeno depositado subcutanea o intramuscularmente pueda ponerse en contacto con las células stem circulantes y producir un tumor local de ellas, mas bien que del tejido conjuntivo adyacente.

Para el estudio *in vitro* de la acción de los agentes externos sobre las células stem, sería de utilidad aislarlas, posiblemente por los procedimientos mecánicos o fisiológicos que se mencionan en este trabajo. El transplante de células stem de diferentes lugares topográficos, después de su exposición *in vitro* a los carcinógenos, podría ser de considerable interés en la inducción de cancer.

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PLATE 11

FIGURE 1.—Many mononuclear cells and red blood cells from layer *B* of bone marrow cells in a centrifuged bovine serum albumin gradient (8).

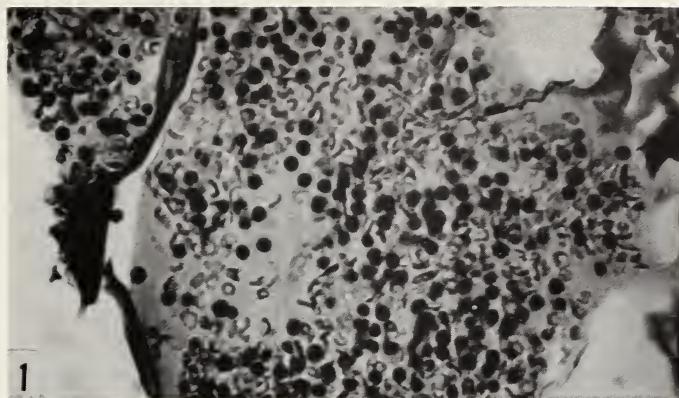
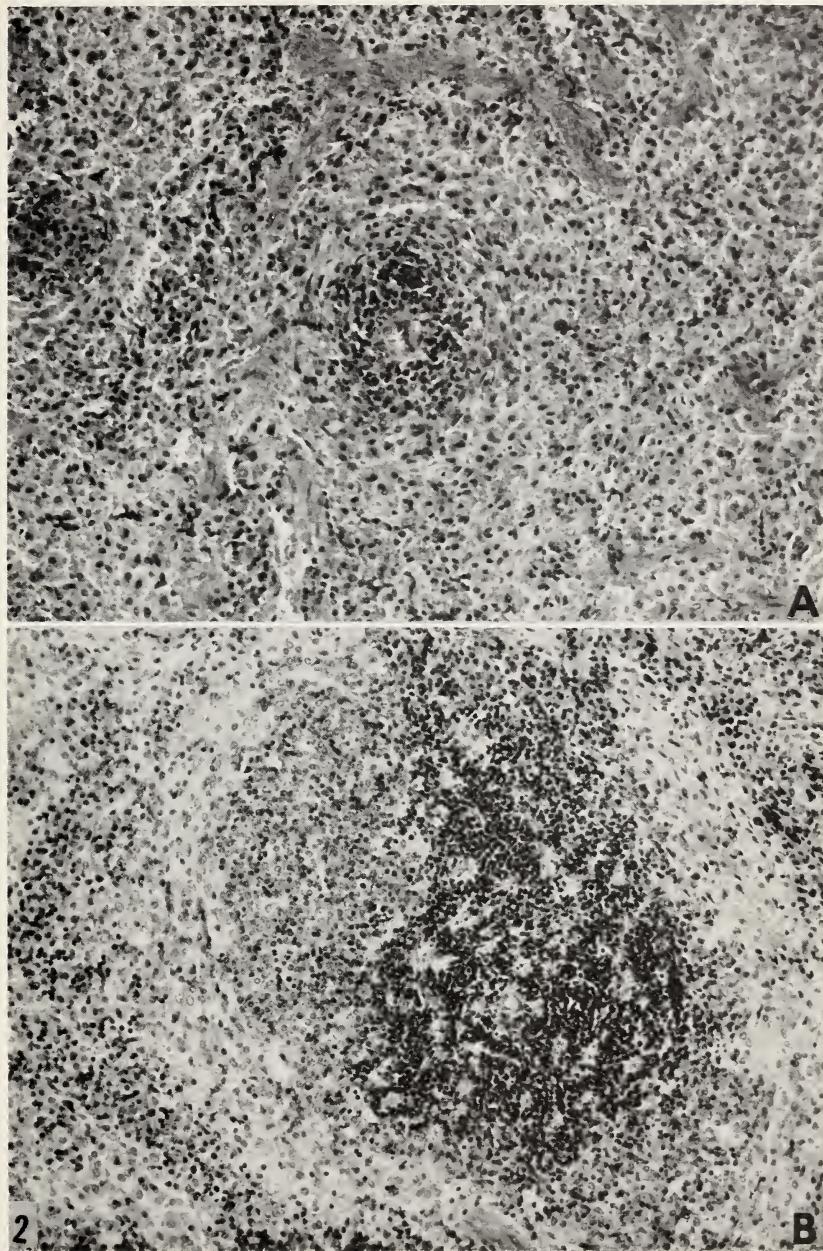


PLATE 12

FIGURE 2.—White pulp of mouse spleen. $\times 180$. (A) Irradiated, untreated BC₃F₁ male, 10 days after 950 r of X rays. (B) Irradiated BC₃F₁ male, 10 days after 950 r of X rays and infusion of 125 million isologous lymph node cells.



DISCUSSION

Leblond: You mentioned an attempt to identify stem cells in various tissues. Would you elaborate on this point?

Goodman: Of course, we would like to know what they are. We have done some labeling in the peripheral leukocyte experiments and found only primitive-looking cells, maybe lymphoblasts, labeled. This was a very small percentage as Leblond, Fliedner, and others have found. What the stem cells really look like, I cannot say; they could be almost any nucleated type found there. We know that there are fewer of these cells than there are in bone marrow, but we have made no attempt to correlate morphology with counts.

Hoecker:* It has been repeatedly claimed that the lymphocytes can be observed to undergo metamorphosis and to differentiate into all other types of blood cells (Review, in O. A. Trowell, *Int Rev Cytol* 7: 249-261, 1958). Did you say that small lymphocytes are just something which exists there but do nothing?

Goodman: No. I said our transplantation studies using lymph node cells, a great number of which were small lymphocytes, gave rise to nothing but lymphatic tissue cells. They gave rise to no red cells and no granulocytes. Of course, there could be two kinds of small lymphocytes, as has been postulated, but there are clearly some cells that look like small lymphocytes of lymph nodes but behave differently. All I can say from our studies is that lymph node cells do not make red cells and granulocytes insofar as we have tested them. I think Carstairs has done a good experiment showing some kind of transformation, and certainly Gowans and Porter and Cooper have demonstrated a kind of transformation; but this is not to say that small lymphocytes are multipotential, by any means.

Upton: Concerning attempts at Oak Ridge to identify stem cells, I feel impelled to mention some work we have done in collaboration with Dr. G. Cudkowicz in our group, Dr. L. H. Smith in Dr. Congdon's group, and Dr. W. L. Hughes at Brookhaven (Cudkowicz *et al.*, *Proc NY Acad Sci USA*, in press). The system is complicated. We who are studying leukemogenesis would like of course to know what cell undergoes the neoplastic transformation, and we were fascinated by studies of Till and McCulloch (*Radiat Res* 14: 213, 1961) in Toronto, in which marrow cells transplanted into a lethally irradiated recipient were found to set up nodular proliferative foci in the recipient's spleen. It was suggested that these foci were clones derived from single stem cells. The system we used was one in which a recipient mouse was irradiated and injected with marrow cells. At intervals, we harvested the cells that were grown in the recipient marrow, and injected them into a second recipient. The ability of the cells to form colony nodules in the spleen of the second recipient was determined.

Hoecker: Is this an isogenic test?

Upton: Yes. To continue, what we were doing was to assay the growth of non-irradiated cells in the first recipient, as judged by their colony formation in the second recipient. As it turned out, the optimal number of cells to produce countable colonies fell within a narrow range, hence we thought, following work by Hughes' group (Gitlin *et al.*, *Science* 133: 1070, 1961), that if we used a γ -emitting DNA precursor it might simplify the assays. So, in subsequent studies we used iododeoxyuridine ($I^{131}UdR$) and counted the percentage of the injected label incorporated in the recipient's spleen.

We found that the percentage of injected $I^{131}UdR$ appearing in the recipient's spleen was indeed a linear function of the number of donor cells injected over a rather wide range of cell dose. However, it turned out that after 5 days' growth in the first recipient, a far larger number of nucleated marrow cells were required to produce a given number of spleen nodules, or a given uptake of the labeled DNA precursor, which suggests that if this response measured stem cells, there had been some reduction in the stem cell pool during the 5 days of growth in the first recipient.

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In one treatment of our data, percentage of uptake of injected $I^{131}UdR$ in the second recipient's spleen was normalized in relation to duration of sojourn in the first recipient, and, as mentioned before, 5 days after introduction into the first recipient, at a dose of 10^7 cells, there were fewer stem cells demonstrable than in nonpretransfused marrow.

In time, the stem cell pool appeared to be restored to normal, and this restoration was more rapid when a larger number of cells was injected into the first recipient. Since most of the nodules in the spleen—at the time we made the assay—were comprised of granulocytopoietic or erythropoietic elements, we speculated that possibly a myeloblast or an erythroblast, cells which can be recognized microscopically on differential smears of the marrow, might show a change in frequency within the injected material corresponding to the change in stem cell capacity. But it turned out that the content of erythroblasts and myeloblasts actually increased before settling down to normal, which indicated clearly that there was no correlation between the content of these recognizable precursors and the response of the recipient spleen.

The only kind of cell which we have been able to find in our limited differential counts thus far, showing the corresponding change in frequency in the marrow, is a small lymphocyte-like cell.

This work inclines us to speculate that a lymphocyte-like cell in the marrow may indeed be the stem cell that we have been talking about. In autoradiographs of marrow in a recipient several days after inoculation, we find labeling with tritiated thymidine over the kind of cell we have been talking about—a small- or medium-sized lymphocyte. The cell tends to label rather heavily in relation to other types.

Leblond: Is a truly small lymphocyte smaller than this labeled cell?

Upton: I am disinclined to attempt a hard and fast differentiation between small, medium, and large lymphocytes. Since cells of this morphology label relatively rarely in normal marrow, it is of interest to find them labeling more often in marrow of the irradiated recipient. We heavily label small lymphocyte-like cells. Larger cells, perhaps transformed lymphocytes in the nomenclature that Dr. Goodman has used, are also seen to be relatively heavily labeled. These cells tend to occur in large numbers somewhat later after inoculation than the smaller cells of lymphocyte morphology.

I think, in summary, it is too early for us to conclude that we have, in fact, a pluripotential cell. I think it is quite clear, as Dr. Goodman suggested, that there are different kinds of lymphocytes. Those in the marrow, perhaps because of their location, may be stem cells; whatever they do elsewhere, they may lose their ability to differentiate in more than a single direction, as do the intestinal crypt cells moving up on the villus.

Goodman: There is, of course, a vast literature on the small, round cell found in the bone marrow, and Yoffey and Courtice (*Lymphatics, Lymph and Lymphoid Tissue*, Cambridge, Harvard Univ. Press, 1956) cite some of it. However, there is considerable doubt that this small, round cell is identical with the small lymphocyte found in lymph nodes or as the major cellular constituent of thoracic duct lymph. If it were identical, one would see hematopoiesis from small lymphocytes when they are transplanted into heavily irradiated mice. No one has been able to find this. There is evidence from different labeling patterns that the two cells are different (J. C. Schooley *et al.*, in *Kinetics of Cellular Proliferation*, New York, Grune & Stratton, 1959), and electron microscopically there appear to be distinct differences (J. C. Schooley and T. L. Hayes, unpublished observations). The small, round cell of the marrow may well be the ultimate hematopoietic stem cell, as J. C. Schooley's and Karl Giger's work (U.C.R.L. Report 10682, Fall, 1962) would suggest, but it is probably not a small lymphocyte.

Kaplan: I would like to offer one alternative interpretation of Upton's data. It seems to me that the demonstration that $I^{131}UdR$ -uptake in the spleen is linear with cell dose is very useful, but the proportion of $I^{131}UdR$ that goes in will be a reflection of the balance between endogenous and exogenous thymidine incorporation and the $I^{131}UdR$ is simply replacing the exogenous thymidine. Unless experiments show convincingly that the access of exogenous thymidine to cells is constant as a function of time after

residence in the intermediate host, I do not think that you can conclude that numbers of stem cells are necessarily different. It is equally possible that the very young stem cells of the 5-day harvest are, for some reason, selectively less able to utilize exogenous thymidine and therefore exogenous $I^{131}UdR$. You would get an apparent, but spurious decrease in the number of stem cells where, in fact, there is no decrease.

Upton: I heartily agree with Dr. Kaplan that a number of additional experiments and data are necessary before we can make a final interpretation. I hope I have not given the impression that I am suggesting that the lymphocytes, any and all lymphocytes, are stem cells. I think that the lymphocyte is a generic term, and has been a stumbling block to hematologists. What I would suggest is that a cell in the marrow, having the morphology of the lymphocyte, seems to be a good candidate for the stem cell. I do not imply that any cell anywhere else in the body, certainly not in lymph nodes, has similar biologic properties; I think the evidence is very clearly against it.

Hodgson: Dr. Upton, did I understand correctly that the stem cell pool was decreased or was this a decrease only in the proportion of stem cells for a given population—if you have a case with hyperactive erythropoietic or myelopoietic marrow, you may have a smaller proportion of stem cells in a given cell sample, but the total stem cell number in the mouse may be conserved or even increased.

Upton: We used two assays: 1) the ability of the transfused marrow cells to promote uptake of $I^{131}UdR$ in the recipient's spleen, and 2) the ability of the transfused cells to form colonies in the recipient's spleen. Both types of assays gave comparable results. This indicates the kind of measurement used; I am not sure if that satisfies, or answers, the question.

Hodgson: You mentioned the establishment of efficiency of a given number of cells, which indicates that within a given number of cells there is a certain number of stem cells. In the whole animal the stem cells may have increased, yet the sample of marrow injected may have a smaller proportion of stem cells. Your procedures measure stem cell concentration, not absolute levels in the whole animal.

Kaplan: I believe that Till and McCulloch presented rather good evidence for considering these nodules in the spleen as derived from a single cell, and therefore, clonal in origin. This, then, furnishes a useful technique for trying to evaluate the capacity of an individual cell to produce a diversity of cell parts. Dr. Michael Feldman in Israel has been working with the Till and McCulloch system. He told us that they had been excising spleen nodules produced in this way, making fresh suspensions, and reinjecting these again into lethally irradiated recipients. By the third or fourth serial reinjection of a given spleen nodule, they got pure cultures of either erythropoietic or myelopoietic nature, but not both.

Erythropoiesis, a Model for the Study of Factors Affecting Cell Proliferation in the Mammal^{1, 2}

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SUMMARY

The advantages of erythropoiesis as a model system for the study of factors affecting cell proliferation are discussed. Experimental results of studies carried out with this system are presented. The dose-response curve to erythropoietin in irradiated plethoric mice given injections of bone marrow is different from that of nonirradiated plethoric mice. The maximum effect depends on marrow dose, and the dose producing half maximum effect is 6 times greater than in nonirradiated recipients. Assays of radiosensitivity of erythropoietically competent stem cells have been carried out by two methods: In one the ability of the irradiated cells to repopulate erythropoiesis in a lethally irradiated host is evaluated; in the other the capacity of the animals' own surviving stem cells to repopulate is measured.

Fractionation of γ -ray dose had a marked effect on recovery of erythropoietic function after irradiation. Recovery was maximum when doses were separated by a 5-hour interval. "Survival" curves for erythropoietic function after γ -ray doses varied with the state of erythropoiesis at the moment of irradiation: the greater the level of erythropoiesis, the larger the shoulders on the curves. Recovery of erythropoietic function after irradiation in animals with high levels of endogenous erythropoietin showed a biphasic response, with an early abortive rise at day 6 and final recovery at day 18 at which time erythropoietic function was recovered in irradiated animals with low endogenous levels of erythropoietin.—Nat Cancer Inst Monogr 14: 169-184, 1964.

ERYTHROPOIESIS OFFERS certain advantages as a model system for studying factors affecting cell proliferation in the whole mammal. The final cellular product, the red blood cell (RBC), has a lifespan measured in months, which it lives out in one body compartment, the circulating blood. Measures of total RBC population can thus be made with satisfactory precision. The precursors of the RBC are highly specialized cells, a major portion of their biochemical machinery being dedicated to the synthesis of one protein, hemoglobin (Hb). This protein is easily measured

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

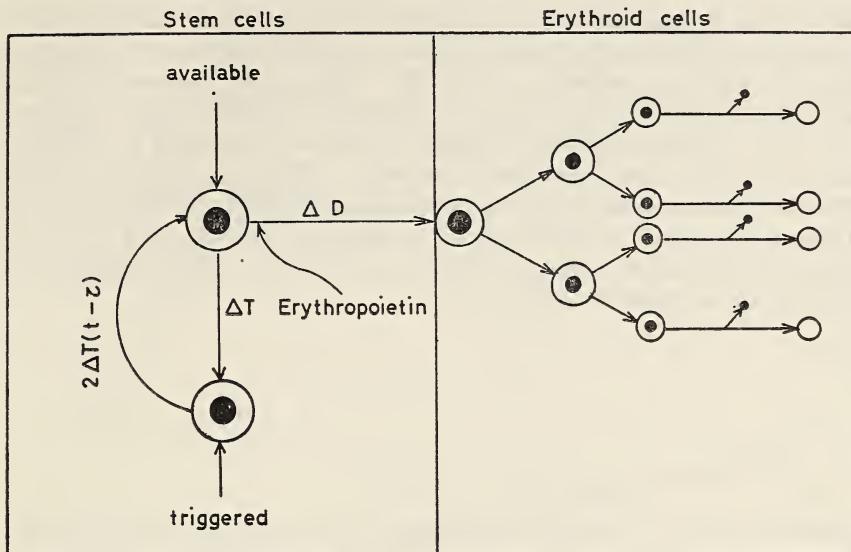
² Supported in part by a grant from the Comisión de Investigación de la Escuela de Medicina, Universidad de Chile, and a grant, A.T. (30-1)-2488, from the U.S. Atomic Energy Commission.

and its rate of synthesis can be inferred from measurements of the uptake of Fe^{59} or labeled amino acids. An average of about 2 cell divisions occur during the process of transformation of an erythroblast into a non-nucleated RBC (1). These cell divisions are notable because a reduction in the information content of the nucleus takes place (2) as well as a reduction in the biochemical potentialities of the cell (3). The Hb-forming RBC precursors are not self-maintaining, but are cells destined to lose their capacity for division and finally their nucleus. The RBC precursors can be conceived as the erythropoietic proper compartment (4). This compartment is replenished by differentiation of "stem cells," self-maintaining nonspecialized, non-Hb-synthesizing cells, which can be stimulated to differentiate into RBC precursors. This differentiation of stem cells into RBC precursors is brought about by a humoral factor, erythropoietin (5). The effect of this material has been clearly shown in studies of plethoric mice (hematocrit, approximately 70%), whose hematopoietic tissues are devoid of erythroid precursors (5). Appearance of RBC precursors follows injection of microgram quantities of erythropoietin (5). The autoradiographic studies of Alpen (1) indicate that the newly formed RBC precursors—capable of Fe^{59} -uptake—arise from cells that do not take up Fe^{59} .

Not all stem cells are available for differentiation into RBC precursors. This is concluded from failure to exhaust the stem cell pool in experiments in which exogenous erythropoietin (6) was injected chronically, and in experiments in which stem cell number was depleted by irradiation, and high levels of endogenous erythropoietin were maintained for long periods (7). Lajtha has suggested that only stem cells not in cell cycle can differentiate while cells in cell cycle—triggered cells—cannot (6). Lajtha also has postulated that stem cell number is regulated by a feedback mechanism by which the number of stem cells entering the cell cycle are a function of the reciprocal of the number of stem cells present. In the steady state for each stem cell that differentiates, another is triggered into division cycle, presumably into S phase—DNA synthesis. These postulated relations are presented in text-figure 1.

This paper presents results of experiments designed to study effects of erythropoietin and irradiation on the erythropoietically competent stem cell population. Data are presented on: 1) use of dose-response curves to erythropoietin in the assay of stem cell number and variations of affinity of stem cells for erythropoietin; 2) use of the lethally and sub-lethally irradiated mouse to assay stem cells to obtain information on radiosensitivity, the protective effect of anoxia, and the influence on γ -ray-dose fractionation; and 3) the influence of increased stem cell activity on radiosensitivity and the effect of high endogenous erythropoietin levels on recuperation after a single dose of irradiation.

In all these studies, the state of erythropoiesis, as estimated by Fe^{59} -uptake, is assumed to reflect changes in the erythropoietically competent stem cell pool.



TEXT-FIGURE 1.—Differentiation of stem cells into erythroid cells.

DOSE-RESPONSE RELATIONSHIP FOR ERYTHROPOIETIN

Stem cells are postulated to be targets for erythropoietin. If it is assumed, as Stetten (8) has suggested, that concentration of hormone (H) is proportional to dosage and the response is proportional to hormone

bound to target (HT), a plot of $\frac{\text{dose}}{\text{effect}}$ vs dose, or effect vs effect/dose or

$\frac{1}{\text{effect}}$ vs $\frac{1}{\text{dose}}$, should give straight lines. From these straight lines, esti-

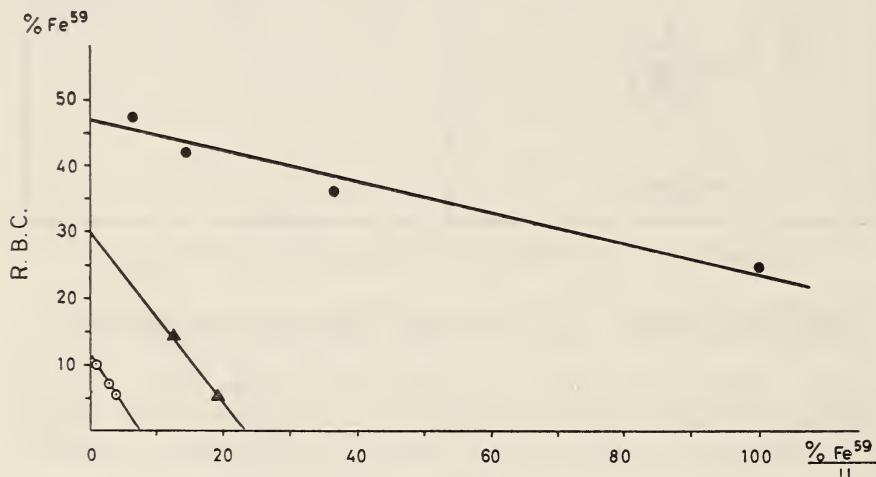
mates of the maximum effect (E_{\max}) and K, the dissociation constant for HT, can be obtained. In this type of analysis, the maximum effect is a measure of the total number of targets (stem cells), and the reciprocal of K is an estimate of the affinity of stem cells for erythropoietin. Our

previous studies (9) indicated that the straight-line relationships for $\frac{1}{\text{effect}}$

vs $\frac{1}{\text{dose}}$ hold for erythropoietin. Our recent studies show that the other

two relationships also hold. A practical plot is that of effect vs effect/dose (10). The intercept of the line obtained gives an estimate of the maximum effect. Text-figure 2 shows dose-response relationship for erythropoietin in plethoric LAF₁ mice. The top line represents the relation observed in nonirradiated mice. The maximum effect is 47 percent uptake of Fe⁵⁹ (24 hr) in RBC, and K = 0.25 units (U) of erythropoietin. The next two lines represent results of experiments carried out with lethally irradiated

plethoric mice that had received different doses of bone marrow. The top curve represents mice that received the dose of 10^7 and the bottom curve, those which received 5×10^6 cells. The marked decrease in maximum effect is clear. It is also worthy of notice, that the slope is steeper, $K = 1.3$ U. Thus, in lethally irradiated animals the affinity for erythropoietin is smaller than in nonirradiated mice. This conceivably could be explained as an increase in factors other than erythropoietin which compete for the same target. If stem cells are multipotential, this could reflect an increase in irradiated animals of factors stimulating differentiation into myelopoietic or thrombopoietic cells.

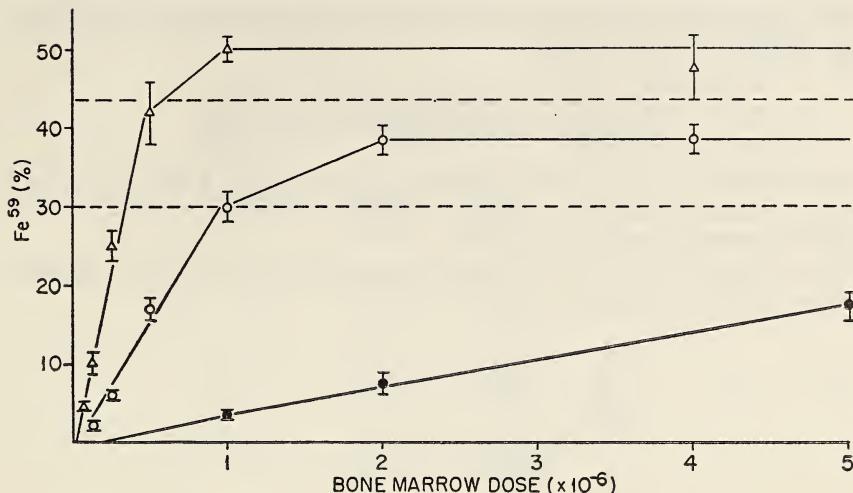


TEXT-FIGURE 2.—Ordinate: Fe⁵⁹-uptake of red blood cells (RBC) in: plethoric mice (●), irradiated (950 r) plethoric mice injected with 10^7 bone marrow cells (▲), and 5×10^6 bone marrow cells (○). Abscissa: ratio of Fe⁵⁹-uptake to erythropoietin dose in units. Irradiation carried out at 0 time, followed by bone marrow injection on the same day, RBC transfusion next day. Erythropoietin injected 3d day, Fe⁵⁹ on 5th day, and RBC sampled on 6th day.

Although more investigation is needed, it appears that this type of approach to the stem cell problem may give useful information on stem cell pool size and affinity of stem cells for erythropoietin under different experimental conditions. The results presented also suggest caution in assuming (11) that the response to a given dose of erythropoietin in the irradiated plethoric mice is a good measure of stem cell number.

A METHOD OF *IN VIVO* CULTURE WITH TRANSPLANTATION

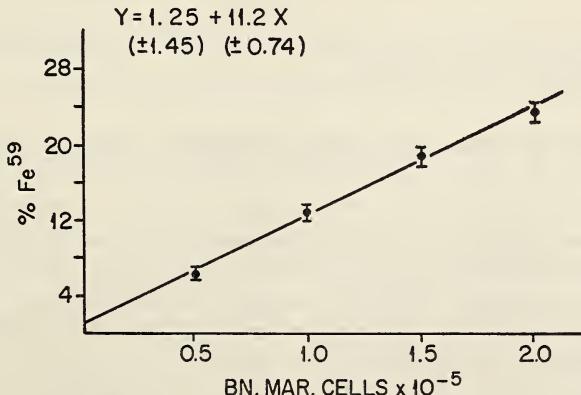
Text-figures 3 and 4 show the experimental basis for the assay when lethally irradiated mice are used as *in vivo* cultures for transplanted stem cells (12). Fe⁵⁹-uptake by RBC of host mice injected with the tracer at different times after irradiation is a function of bone marrow dose. For



TEXT-FIGURE 3.—Twenty-four-hour erythrocyte Fe^{59} -uptake in lethally irradiated LAF_1 mice given isologous bone marrow. Fe^{59} injected 5 days after irradiation (●—●), Fe^{59} injected 7 days after irradiation (○—○), and Fe^{59} injected 9 days after irradiation (△—△). Dashed lines through 30 and 43.5 percent Fe^{59} represent the uptake of 20 normal mice and of mice injected with 12.5 cobalt units of urinary ESF.

Fe^{59} -uptakes between 0 to 30 percent, the relation is a linear one. The steepest line, relating Fe^{59} -uptake to marrow dose, is that obtained when Fe^{59} is injected on day 9 and covers the range of 5×10^4 to 5×10^5 nucleated bone marrow cells.

The method outlined has been used to study the radiosensitivity of erythropoietically competent stem cells (13). Since RBC Fe^{59} -uptake is directly proportional to stem cell dose, the RBC Fe^{59} -uptake observed



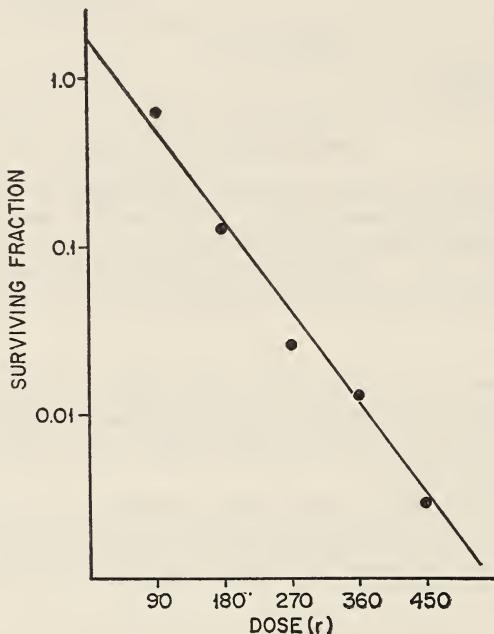
TEXT-FIGURE 4.—Relation between bone marrow (Bn Mar) dose and percent of uptake of Fe^{59} in erythrocytes. Vertical lines indicate the mean \pm one standard error. Regression was calculated from individual values from 62 mice.

after injection of irradiated cells can be assumed proportional to the viable cells present. Thus the ratio:

$$\frac{\text{Percent Fe}^{59} \text{ RBC/No. irradiated cells}}{\text{Percent Fe}^{59} \text{ RBC/No. nonirradiated cells}}$$

is an estimate of the surviving fraction. Text-figure 5 and table 1 show survival curves of *in vivo* irradiated cells from (C57BL \times C3H)F₁ mice and the protective effect of anoxia.

The values for D₃₇ are 71 r for cells irradiated *in vitro* and *in vivo*; the extrapolation number is 1.75.



TEXT-FIGURE 5.—Surviving fraction of *in vitro* irradiated erythropoietically competent cells as a function of X-ray dose (r).

METHOD OF *IN VIVO* CULTURE WITHOUT TRANSPLANTATION

In this experimental setup, the mouse receiving a sublethal dose of irradiation was used as a "culture medium" for those of its own stem cells escaping radiation damage and able to proliferate. Fe⁵⁹ was injected 8 days after irradiation, and RBC sampled 24 hours later. Text-figure 6 shows 24-hour RBC Fe⁵⁹-uptake in (C57BL \times C3H)F₁ female mice as a function of 0.66 Mev γ -irradiation (7.6 r/min) dose. The relation is exponential with a D₃₇ of 81 r. The difference in D₃₇, for experiments with the method of *in vivo* culture with transplantation (see previous section) with 250 kvp X rays, can conceivably be explained by difference in dose rate and a lower RBE of the 0.66 Mev γ -rays.

TABLE 1.—Estimated surviving fraction of erythropoietically competent cells of marrow irradiated *in situ*, *in vivo*, and of marrow irradiated *in situ* after death

X-ray dose (r)	<i>In vivo</i>		After death	
	Cell dose	Surviving fraction*	Cell dose	Surviving fraction*
90	6×10^5	0.3660 ± 0.022	2×10^5	0.654 ± 0.07
	4×10^5	0.2980 ± 0.019		
	2×10^5	0.2900 ± 0.046		
180	1.2×10^6	0.1710 ± 0.0075	4×10^5	0.517 ± 0.034
	8×10^5	0.1760 ± 0.0171		
	2×10^6	0.1650 ± 0.0117		
270	6×10^6	0.0417 ± 0.00228	2×10^6	0.085 ± 0.014
	4×10^6	0.425 ± 0.00350		
	2×10^6	0.0315 ± 0.00125		
360	2.4×10^7	0.0098 ± 0.00075	2×10^6	0.085 ± 0.014
	1.6×10^7	0.0066 ± 0.00061		
	8×10^6	0.0076 ± 0.00078		
450	6×10^7	0.0029 ± 0.000253	2×10^6	0.085 ± 0.014
	4×10^7	0.0024 ± 0.000164		
	2×10^7	0.0027 ± 0.000440		

* \pm Standard error.

Table 2 shows the effect of γ -ray-dose fractionation on RBC Fe^{59} -uptake in (C57BL \times C3H)F₁ mice. The decrease in the effect of a given total dose when the interval between doses is less than the period of division, delay imposed by the first dose, has been termed "recovery" by Elkind (14). This effect has been observed in cultured mammalian cells (14), and also by *in vivo* methods, when the spleen-nodule counting technique was used (15). However, in our hands this technique did not show "recovery" in a clear-cut fashion, while, as table 2 illustrates, the RBC Fe^{59} -uptake method did. Maximum recovery was observed with a 5-hour interval between doses.

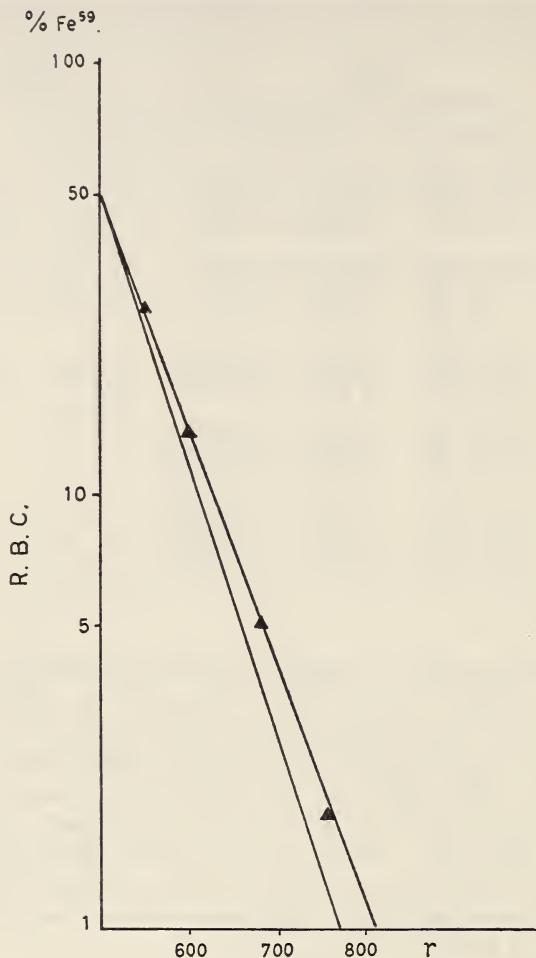
EARLY DEPRESSION OF ERYTHROPOIETIC FUNCTION

Text-figure 7 shows the depression of uptake of Fe^{59} by erythropoietic tissue in A \times C rats as a function of γ -ray dose. One group of rats re-

TABLE 2.—Effect of interval (Δt) between γ -ray doses on "recovery" of erythropoiesis

Dose (r)	Irradiation time (min)	Δt (hr)	RBC (% Fe^{59})*	Spleen nodules
550	72	0	26.8 ± 3.0	10.5 ± 1.2
600	79	0	14.5 ± 1.5	8 ± 1.0
600	40 + 39	2	27.9 ± 3.9	10 ± 1.0
600	40 + 39	5	47.4 ± 3.7	12 ± 2.0
600	40 + 39	8	41.2 ± 4.4	12 ± 2.0

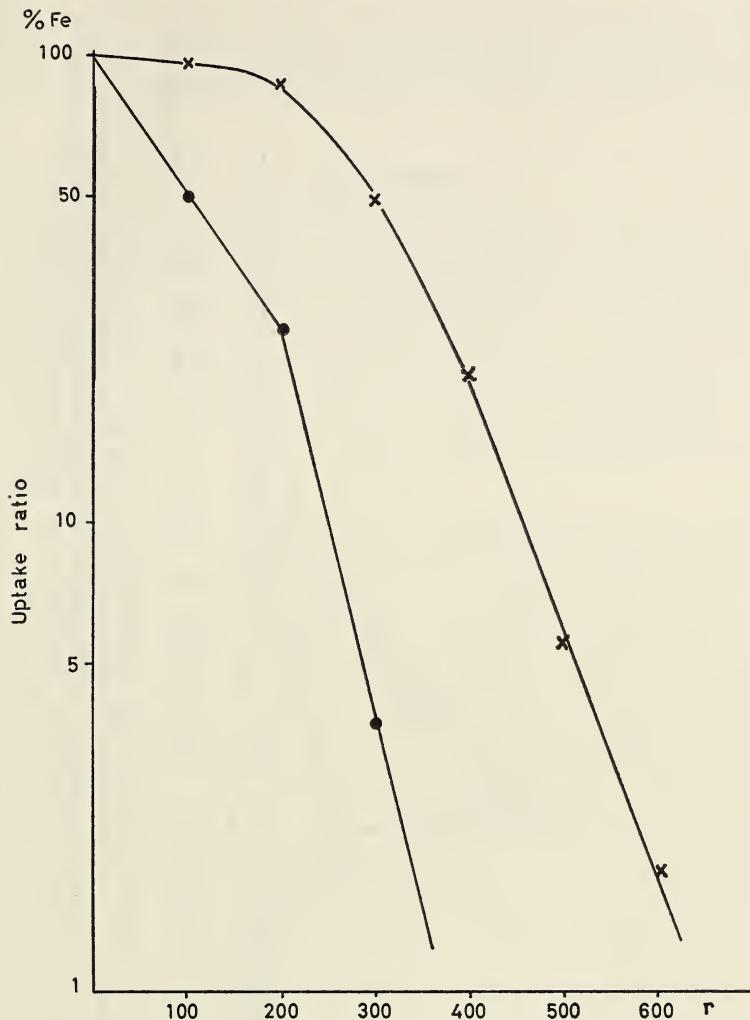
*Twenty-four-hour uptake at 8 days.



TEXT-Figure 6.—Twenty-four-hour Fe^{59} -uptake in RBC of mice γ -irradiated 8 days before tracer injection (\blacktriangle). Continuous line (—) represents survival curve shown in text-figure 5.

ceived the dose when erythropoietic activity of bone marrow was at a maximum, as a consequence of phenylhydrazine treatment. The other group had normal erythropoietic function at the time of irradiation.

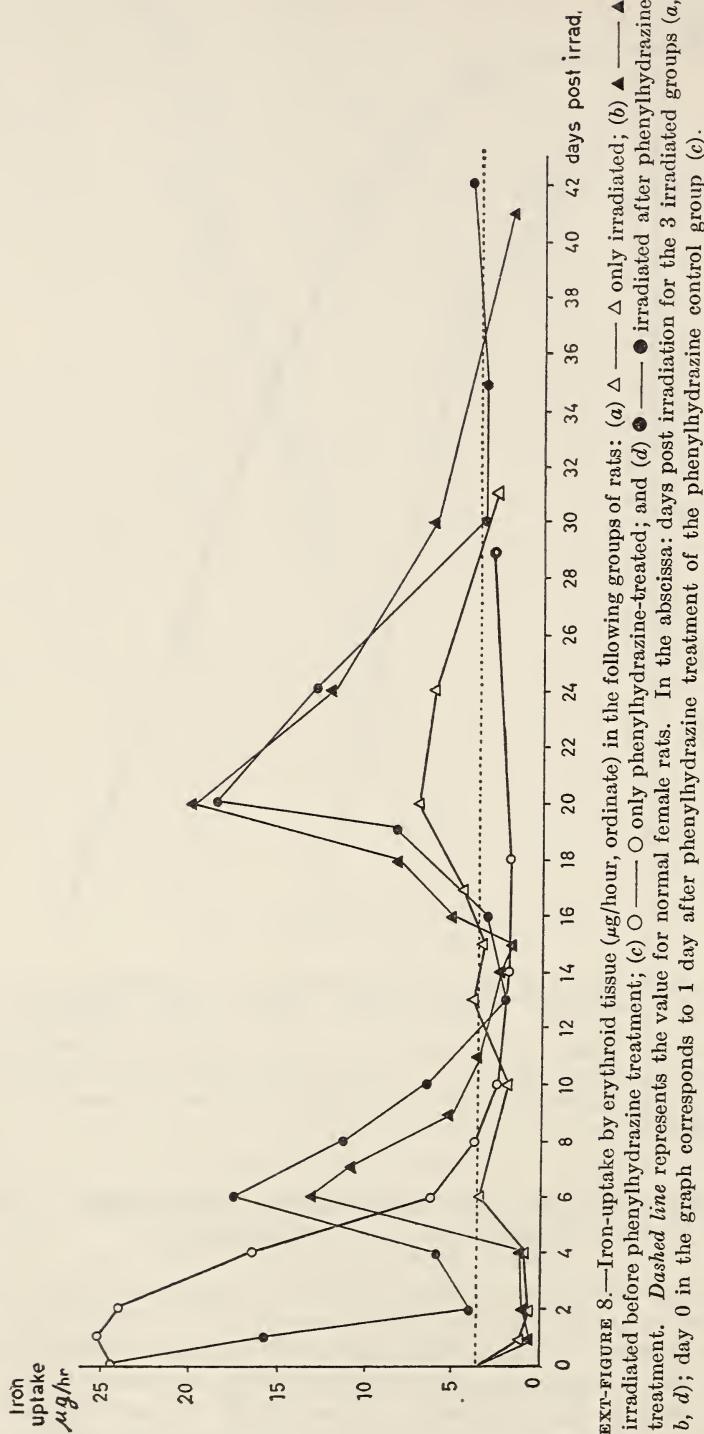
The ratio of irradiated to control-iron-uptake by erythropoietic tissue, measured 2 days after irradiation, is plotted versus γ -ray dose. Iron-uptake values were corrected for the small fraction of uptake which is not affected by irradiation. There is a clear difference between the two curves. The curve of the rats irradiated while erythropoietic function was at a maximum has a large shoulder; 100 r produces no reduction, while in the normal rats Fe^{59} -uptake drops to 50 percent. The exponential portion of the curve shows a D_{37} of 80 r.



TEXT-FIGURE 7.—Ratio of iron-uptake by erythropoietic tissue of irradiated rats to that of nonirradiated rats: X — X rats irradiated ($\text{Cs}\gamma$) when erythropoiesis was at a maximum (phenylhydrazine treatment); ● — ● rats irradiated when erythropoiesis was normal.

RECUPERATION OF ERYTHROPOIETIC FUNCTION AFTER A SINGLE DOSE OF IRRADIATION

Text-figure 8 shows the effects of high endogenous levels of erythropoietin on recovery of erythropoietic function in rats after a dose of 500 r of Cs^{137} , 0.66 Mev γ . Rats with high endogenous levels of erythropoietin show an abortive rise of iron-uptake by erythropoietic tissue to a maximum on the 6th day after irradiation. Uptake falls thereafter to low levels, to rise to a new maximum at about day 20, coinciding with the rise in



TEXT-FIGURE 8.—Iron-uptake by thyroid tissue ($\mu\text{g}/\text{hour}$, ordinate) in the following groups of rats: (a) \triangle — \triangle only irradiated; (b) \blacktriangle — \blacktriangle irradiated before phenylhydrazine treatment; (c) \circ — \circ only phenylhydrazine-treated; and (d) \bullet — \bullet irradiated after phenylhydrazine treatment. *Dashed line* represents the value for normal female rats. In the abscissa: days post irradiation for the 3 irradiated groups (a, b, d); day 0 in the graph corresponds to 1 day after phenylhydrazine treatment of the phenylhydrazine control group (c).

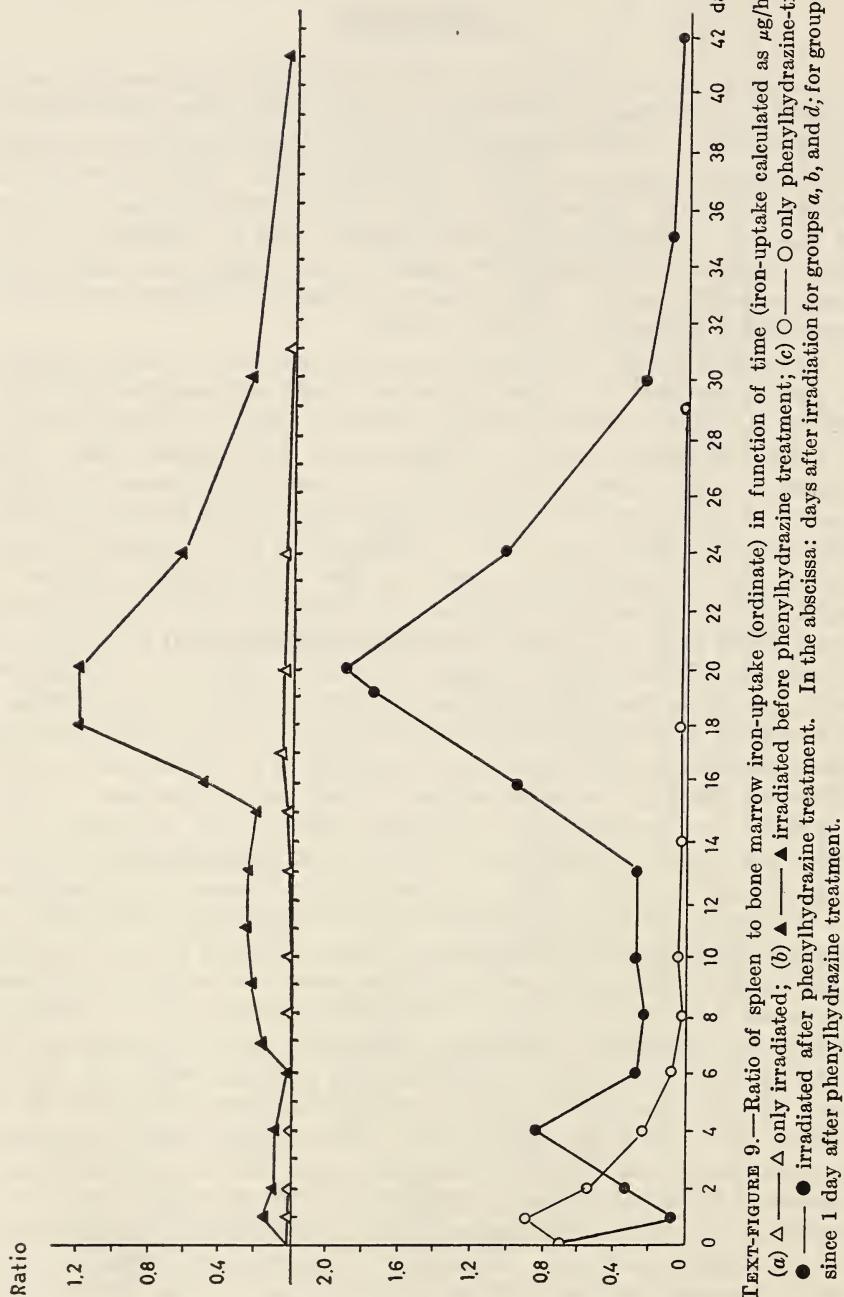
uptake in normal rats and preceding the recovery of normal Hb levels. It is of interest (text-fig. 9) that the spleen plays a predominant part in the final rise in erythropoietic activity, but not in the initial abortive rise.

DISCUSSION

Our data presented here indicate that quantitative information can be obtained on the effects of irradiation on stem cell number and affinity for erythropoietin. Stem cells can be assayed by their capacity to repopulate erythropoietic tissue in lethally irradiated hosts. Survival curves for erythropoietically competent stem cells can be constructed, and estimates of D_{37} and extrapolation number obtained. The clear effects of γ -ray-dose fractionation of RBC Fe⁵⁹-uptake in mice suggest that this method may be useful for the study of factors which can influence "recovery" occurring in intervals as short as 5 hours.

In animals in which erythropoiesis is at a maximum—7 times normal—it can be assumed that many more stem cells are being triggered into cell cycle, and if the mitotic interval remains constant, in the steady state 7 times the normal amount of cells are in the triggered state. Rats irradiated under these conditions show an inactivation curve of erythropoietic function markedly displaced to the right of that for rats irradiated when erythropoiesis was normal. Similar shifts (16) are observed when comparing the inactivation curve, for the erythropoietic response to erythropoietin given after irradiation, of normal mice with that of plethoric mice, which have a completely depressed erythropoiesis at the moment of irradiation. These findings suggest that stem cells in a nontriggered state, available for differentiation, are more sensitive to irradiation; if this were so it would place them in the category of the lymphocytes and spermatogonia in which sensitivity is not related to mitotic activity (17). These results, based on experiments in which early depression of erythropoiesis was the parameter measured, will have to be confirmed by use of the *in vivo* transplantation method to assay radiosensitivity of erythropoietically competent cells as a function of the state of erythropoiesis.

The effects obtained with high levels of endogenous erythropoietin on recuperation after γ -irradiation are not those predicted by the hypothesis (18) which postulates that erythropoietin speeds recuperation by inducing increased differentiation of stem cells and thus reducing their number and indirectly stimulating proliferation. However, as the experimental data forming the basis for this hypothesis were obtained within the first 10 days of irradiation, the increase in erythropoiesis may conceivably be the abortive rise seen in our experiments. This rise may reflect the presence in bone marrow of "partially damaged" stem cell, *i.e.*, cells able to go through a few divisions before dying (16). The "damaged" cells are by definition non-self-maintaining and thus cannot influence final recuperation of the stem cell pool. High levels of erythropoietin could, however, make these cells evident by "flushing" them out into the erythropoietic



TEXT-FIGURE 9.—Ratio of spleen to bone marrow iron-uptake (ordinate) in function of time (iron-uptake calculated as $\mu\text{g}/\text{hour}$). Groups: (a) Δ — Δ only irradiated; (b) \blacktriangle — \blacktriangle irradiated before phenylhydrazine treatment; (c) \circ — \circ only phenylhydrazine-treated; and (d) \bullet — \bullet irradiated after phenylhydrazine treatment. In the abscissa: days after irradiation for groups *a*, *b*, and *d*, for group *c*, time counted since 1 day after phenylhydrazine treatment.

compartment proper, where their presence is detected as a temporary rise in erythropoietic activity. In rats with low levels of endogenous erythropoietin, they would not be so evident. If the observed early rise in erythropoietic function is a reflection of the proliferation of cells, which will ultimately leave no viable daughters, it would indicate that this type of cell is capable of carrying out a specialized function. Information of this type of phenomena in *in vivo* irradiated mammals is lacking (17) and the observation just-described presents a useful model for further study.

The preponderant role played by the spleen in the final erythropoietic recovery in phenylhydrazine-treated rats suggests that this organ may contain more primitive (and relatively more radioresistant) reticulo-endothelial cells which can become stem cells, or that the spleen, as a consequence of its hemolytic activity, secondary to phenylhydrazine treatment, may be a more favorable environment for stem cells that migrate there from bone marrow via the blood.

RESUMEN

Se discute el valor de la eritropoyesis como modelo en qué estudiar los factores que afectan la proliferación celular. Se presentan resultados experimentales obtenidos con este sistema.

Las curvas dosis efecto de ratones, transfundidos con eritrocitos, irradiados e injectados con médula ósea, difieren de las de ratones no irradiados policitemicos por transfusión. El efecto máximo en los ratones irradiados depende de la dosis de médula ósea injectada. La dosis que produce un efecto, la mitad del efecto máximo es seis veces mayor en ratones irradiados que en los no irradiados.

Se han hecho ensayos de células madres eritropoyéticamente competentes utilizando dos métodos: en uno de ellos se valora la capacidad de células irradiadas de repoblar el tejido eritropoyético de ratones letalmente irradiados; en el otro se valora la capacidad de las células sobrevivientes del propio animal irradiado para repoblar su tejido eritropoyético. El fraccionamiento de la dosis de irradiación tiene marcado efecto sobre la recuperación de la eritropoyesis post irradiación. La recuperación es máxima cuando el intervalo entre las dos dosis es de cinco horas.

Las curvas de "sobrevida" de la función eritropoyética después de dosis de irradiación, varían según el estado de la eritropoyesis en el momento de la irradiación. A mayor eritropoyesis las curvas presentan mayor hombro.

La recuperación de la eritropoyesis post irradiación en ratas con tasas elevadas de eritropoyetina endógena se hace siguiendo una curva bifásica. A los seis días se presenta una primera alza abortiva: la recuperación final ocurre al mismo tiempo (18 días) que en animales con tasas endógenas bajas de eritropoyetina.

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DISCUSSION

Lamerton: You carefully used the words "stem cells." In the experiments in which one tests "stem cell capacity," by transplanting bone marrow from one animal to another, is one only measuring erythropoietic-populating ability of erythropoietically competent cells? Have these cells, in fact, any relation to what one normally regards as the stem cell in living animals; that is, the earliest precursors, and the stem cells that Lajtha has talked about in his very hypothetical model? When you transplant, it may not be only the earliest precursors that can have reproductive integrity, and can become ancestors of another population, but it could well be that cells further along the line of differentiation dedifferentiate and themselves become ancestors. So the question is really, to what extent do the results on transplantation of bone marrow represent what we regard as a stem cell population?

Hodgson: In the stem cells I have been talking about, erythropoietically competent stem cells, I feel that they are not in early stages of recognizable red cell precursors

because when you transplant leukocytes you get regeneration of erythropoiesis (Blood 19: 702, 1962). In a study of 10,000 nucleated cells which Dr. E. E. Capalbo carried out, he was unable to identify anything that looked like recognizable red cell precursors.

We have also done experiments in which we have transplanted marrow tissue from plethoric mice in which erythropoiesis is markedly depressed. And this tissue repopulates erythropoietic tissue just the same.

Totter:* Experiments have been done in which pernicious anemia patients were kept under oxygen—pernicious anemia patients in relapse—then they were injected with liver extract containing vitamin B₁₂. They did not respond to the injections with the usual reticulocytosis and disappearance of megaloblastosis. Now it is all very comfortable to assume that the failure of response was a depression of erythropoietin, but I cannot quite see how this fits with the scheme you now think of as a change in stem cells.

Hodgson: It is purely hypothetical, but the differentiation of stem cells may depend on the hemoglobin concentration or the oxygen tension. Pernicious anemia patients who have been transfused or breathe 50 percent O₂ show depression of erythropoiesis. In other words, in these patients the control is functioning, and the failure is somewhere else along the line; we do not know exactly what it is. There may be a block in transformation of ribomononucleotides into deoxyribonucleotides, the erythroblasts have a rather high RNA content all during the cycle instead of RNA diminishing rapidly. So what is presumably the case is that when one corrects the anemia or the tissue oxygen lack in pernicious anemia one diminishes the stimulus for differentiation and thus reduces the number of target cells for B₁₂—so one would not expect to end up with a reticulocytosis on giving B₁₂.

Congdon: This matter of abilities and varieties in the cell population is of some interest. In thymus recovery after irradiation and bone marrow transplantation there is a biphasic recovery curve that we have observed in marrow transplants, and I believe Kaplan has too. Can one put the recovery after this split-dose experiment in terms of numbers of cells? Can you say, this is equivalent to so many million bone marrow cells transplanted, if one were transplanting? Secondly, what is the current status of the site of action of erythropoietin—does it act through the liver?

Hodgson: One can assume, of course, that iron-uptake in the sublethally irradiated animals is in direct proportion to the remaining stem cell number. And that the proportionality constant is like that of lethally irradiated animals which have received a transplant. I really do not know if there is much point in referring to the number of cells recovered. If one uses these assumptions, one would say that 3 times as many more cells recover when one fractions the dose. Lajtha and Vane have suggested that somehow the marrow is dependent on the liver for some factor essential for purine synthesis. If rat marrow is incubated and an acid-soluble chromatograph fraction from a 100,000 \times g supernatant of liver is added, there is a remarkable increase in uptake of formate into adenine of DNA 10 times, and thymine of DNA 3 times. There is also a threefold increase in the specific activity of adenine in the acid-soluble fraction. A group in our laboratory is working on this. We have hoped for sometime to explore the possibility of ESF acting through liver, but have been impeded in trying to clear up what the active liver substance is.

Upton: The demonstration, if I understand you correctly, that the erythropoietically stimulated marrow is significantly more radioresistant than normal is very noteworthy, it seems to me. This could, of course, indicate a greater number of cells at risk, and therefore a greater number surviving a given dose, although the fraction surviving might not be changed; or indeed, it could indicate that erythropoietin had changed the status of the population of stem cells with respect to radiosensitivity.

Hodgson: One can speculate that the stem cells can be in two states: one in which they are available for differentiation into erythroid elements and one in which they are not. A stem cell which leaves the pool by differentiation somehow triggers another stem cell into cycle. Thus if you increase differentiation, by that you are

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increasing the number of cells that are entering the division cycle. In plethoric mice, which show the greatest radiosensitivity, DNA synthesis is remarkably decreased and so is RNA. This may just reflect a change in population. We really do not know quantitatively how important is the erythropoietic tissue versus the nonerythropoietic or lymphatic tissue in reference to DNA synthesis.

Mole: This abortive rise reminded me of the biphasic weight response you can get in rats (Lamerton, Elson, and Christensen, *Brit J Radiol* 26: 510, 1953). Can the drop-off after the initial increase in uptake of iron be just associated with a failure of the animal to eat properly—the nutritional state of the stem cells, rather than any change in their number?

Hodgson: Yes, that is a difficult problem to answer. Some of the animals that received phenylhydrazine after irradiation are really in poor shape at that time. But animals that receive phenylhydrazine before irradiation, and are irradiated when they are at maximum erythropoiesis, are in good condition as far as weight is concerned. I have not checked on food consumption.

Lamerton: Could you say something about the effect of starvation on Fe^{59} -uptake?

Hodgson: The fasted-animal assay, as opposed to the plethoric assay, was used in our laboratory where it is difficult to get large numbers of animals for transfusion. Starving for 72 hours markedly depresses red cell formation. Injections of erythropoietin brings it right back to normal or several times normal, depending on dose, without affecting the fasting condition.

Studies of Cell Proliferation Under Continuous Irradiation¹

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SUMMARY

A study is reported, in young rats, of the response of various tissues to continuous irradiation over a range of dose rates. It has been found that the renewal tissues of the body differ greatly in the dose rate that can be tolerated. For instance, the epithelium of the small intestine can maintain function and a steady cell population (somewhat smaller than normal) at a dose rate as high as 400 rads per day. The bone marrow can maintain a steady state of cell population only at dose rates of 50 rads per day and below, and the testis is far more sensitive to continuous irradiation than the bone marrow. The various tissues also differ in the way in which the normal pattern of cell proliferation is changed under continuous irradiation. At a dose rate of 50 rads per day the bone marrow

showed a considerable increase in proliferation rate, whereas the epithelium of the small intestine demonstrated only a slight increase. Studies made on the red cell system under continuous irradiation indicated that, in spite of a reduction in bone marrow cellularity (including "stem cell" content), red cell production was little different from normal. This suggests a radical change in cell population kinetics, and this is discussed. Studies are also reported on the response of continuously irradiated tissues to stress requiring increased proliferation. The conclusion is that continuous exposure will not necessarily lead to an accumulation of damage affecting the regenerative capacity of renewal tissues, even at quite high dose rates.—*Nat Cancer Inst Monogr* 14: 185-198, 1964.

IN SO FAR AS one regards cancer as a breakdown of the normal relationship between cell proliferation and differentiation in a tissue it is of interest to determine how far a changed pattern of cell proliferation can be maintained without tissue failure. This is the reason for discussing the effects of continuous irradiation in this Symposium.

Continuous irradiation can be regarded as a form of stress which increases the normal rate of cell loss in the proliferating compartment of a

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tissue. If a steady state of cell population and tissue function is to be maintained, and there is good evidence that this can occur in some tissues under continuous irradiation, it can only be achieved at the expense of some change in the normal pattern of cell population kinetics.

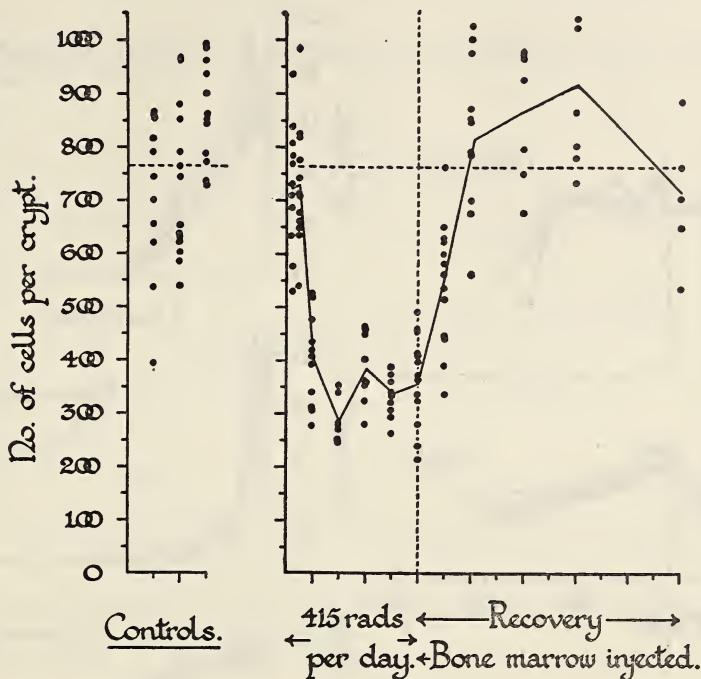
This paper is concerned mainly with the response to continuous irradiation of two tissues, the bone marrow and the epithelium of the small intestine.

The irradiation unit, which has been described previously (1), uses the γ -radiation from a cesium 137 source and allows animals to be irradiated for nearly 24 hours per day at dose rates from 4 to about 400 rads per day. In all our studies we have used young rats, aged 6 to 8 weeks, when put into the unit.

DEVELOPMENT OF A STEADY STATE UNDER CONTINUOUS IRRADIATION

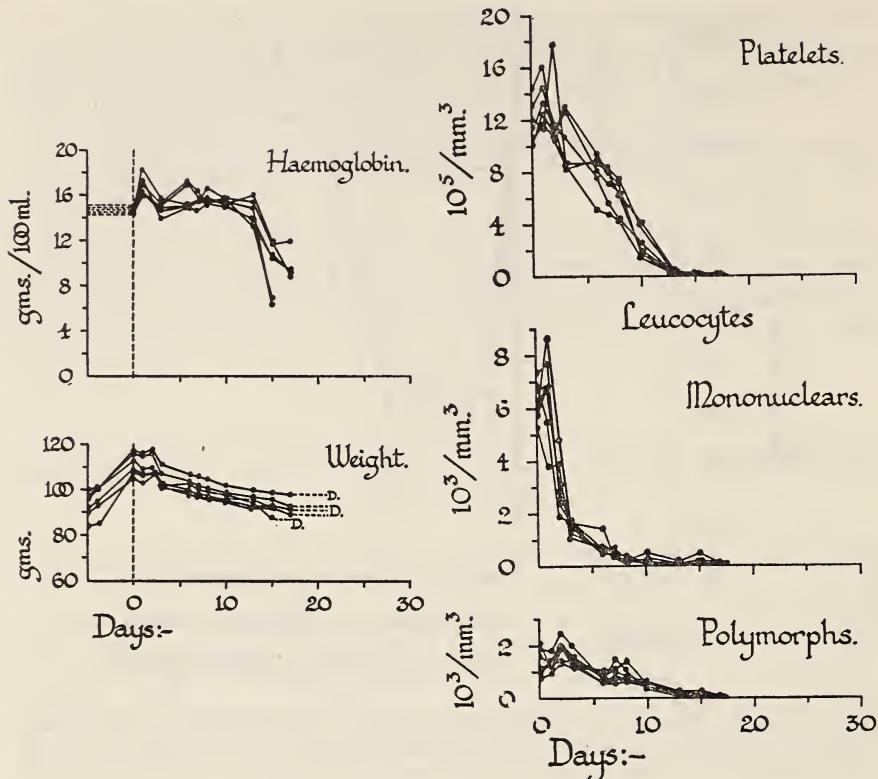
We have confirmed the reports of earlier workers that under continuous irradiation, at least some of the renewal tissues can maintain a steady or near-steady state of cell population for a considerable period, though the maximum dose rates which can be tolerated vary greatly from tissue to tissue. Of the renewal tissues we have investigated, the epithelium of the small intestine is one of the most resistant to continuous irradiation. In the young rat, it will maintain function and a steady cell population (though somewhat smaller than normal) at dose rates of 400 rads per day, until death results from damage to the blood-forming organs. At the other end of the scale is the testis, where progressive cellular depopulation will occur at dose rates of only a few rads per day. Many other of the renewal tissues of the body, including the blood-forming organs, the hair follicles, and cells of the cartilage plate occupy an intermediate position and will maintain a steady or near-steady state of cell population for a long period at dose rates between 50 and 100 rads per day. Within this class there are some very interesting differences. For instance, the erythropoietic system can stand a higher dose rate than either the granulocyte- or platelet-producing systems. At a continuous dose rate of 84 rads per day, radioactive iron studies show red cell production is nearly that normal after 50 to 60 days of exposure, by which time the granulocyte and platelet counts in the blood are at a very low level.

Text-figures 1 to 5 illustrate some of these facts for the gut and the bone marrow. Text-figure 1 shows how the cell count in the crypts of Lieberkühn in the small intestine varies under continuous irradiation at 415 rads per day. These counts were made from squashes of dissected crypts (2). There is a fall to just over 40 percent of the control value within 1 to 2 days and this level is then maintained, but in the experiment illustrated the animal was removed from the unit at 5 days, to study the speed of recovery. Within 2 days the count is back to normal in spite of an accumulated dose of more than 2000 rads.



TEXT-FIGURE 1.—The change in size of the cell population of the crypts of the small intestine during 5 days continuous irradiation at 415 rads per day and during the subsequent recovery following the termination of irradiation.

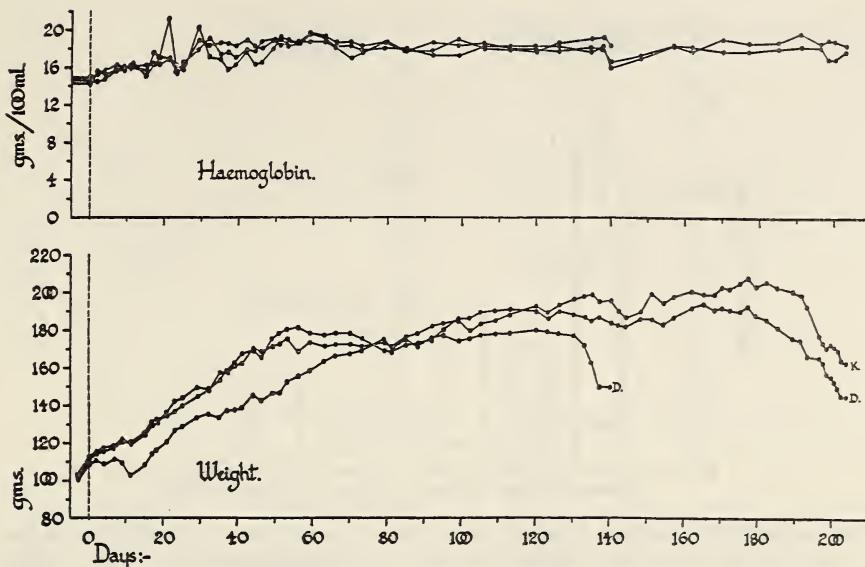
The blood-forming organs, however, show a complete collapse at much lower dose rates. The response of the blood count at a dose rate of 176 rads per day is shown in text-figure 2. Studies made at 84 rads per day give evidence of a transient recovery at about 20 days (3), but it is not until the dose rate is reduced to 50 rads per day that there is a maintenance of blood count for a considerable period. Blood counts at 50 rads per day are shown in text-figures 3 and 4, and it can be seen that, after an initial fall in platelet and white cell counts, a steady value is maintained for as long as 200 days in some cases, by which time the accumulated dose has reached 10,000 rads. However, the blood count is, in many respects, a well-buffered system and a steady blood count does not necessarily indicate a steady state in the proliferative compartment of the system. Dr. P. J. Roylance has studied the bone marrow of rats exposed at 50 rads per day and text-figure 5 shows bone marrow counts for normal animals and for animals after 35 and 105 days irradiation. The lower rectangles represent the dividing precursors and the upper rectangles the nondividing forms. After an initial fall in dividing and nondividing forms it would appear that a steady or near-steady state has been developed in both myeloid and erythroid cell populations, at least between 35 and 105 days from the start of exposure.



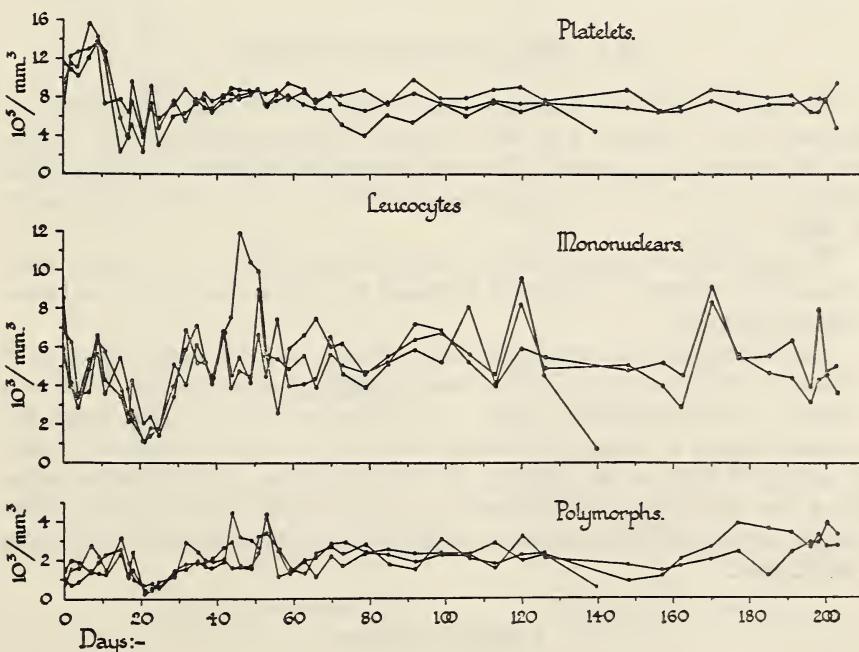
TEXT-FIGURE 2.—Peripheral blood count of hybrid rats irradiated continuously at 176 rads per day. *Left*: hemoglobin and weight; *right*: platelets and leukocytes.

An important question is whether these tissues will show an impaired response to stress which require increased proliferation. The experiment illustrated in text-figure 1 indicates that the gut irradiated at 415 rads per day for 5 days still has the capacity to regenerate rapidly up to its original cell population. So far as the red cell system is concerned we would like to present just one experiment—the effect of removal of one third of the blood volume by cardiac puncture. Text-figure 6 shows the response of the blood count, after 130 days exposure at 50 rads per day, and it can be seen that the recovery of the hemoglobin level is as rapid as in the unirradiated animal. There is, however, an interesting difference in the response of the white count. In the continuously irradiated animals the immediate leukocytosis observed in normal animals is greatly reduced, which suggests a severe depletion of the extracirculatory stores of white cells.

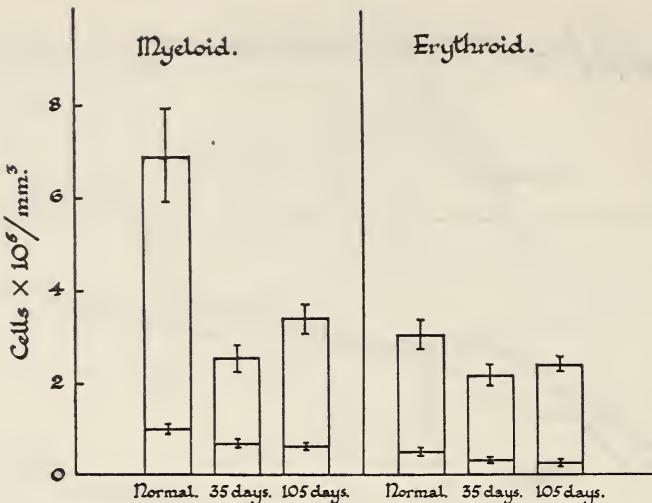
However, these results indicate that continuous radiation exposure does not necessarily lead to an accumulation of damage that will affect the regenerative capacity of the renewal systems of the body, even at quite high dose rates. This is an important conclusion from the viewpoint of



TEXT-FIGURE 3.—Peripheral blood count of hybrid rats irradiated continuously at 50 rads per day: hemoglobin and weight.



TEXT-FIGURE 4.—Peripheral blood counts of hybrid rats irradiated continuously at 50 rads per day: platelets and leukocytes.



TEXT-FIGURE 5.—Absolute numbers (per mm^3) of myeloid and erythroid cells in the bone marrow of rats after 35 and 105 days of continuous irradiation at 50 rads per day and in normal rats. Lower rectangles: dividing precursors; upper rectangles: nondividing precursors.

continuous irradiation used as a tool in the study of cell population kinetics.

CELL PROLIFERATION PATTERN

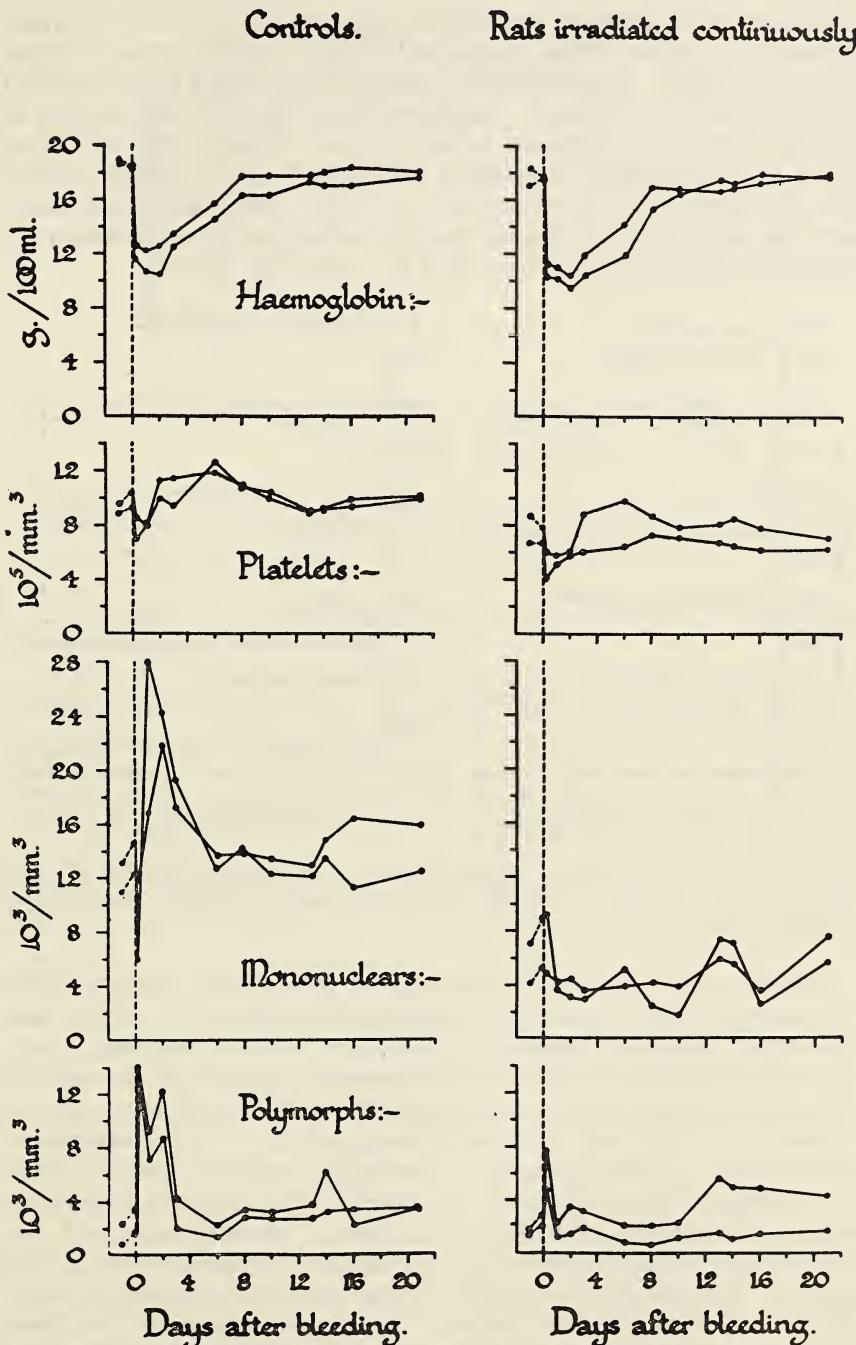
The maintenance of a steady state under continuous irradiation indicates that a balance has been reached between damage and repair; the difference in response of the various tissues indicates a difference in damage produced, or in the speed and efficiency of the repair systems, or in both.

To study the changes in cell proliferation pattern we have used mainly techniques involving the use of tritiated thymidine and high resolution autoradiography.

Autoradiography on specimens taken shortly after a single injection of tritiated thymidine will yield the "labeling index," which can be taken as a measure of cell proliferation rate. Any change in the labeling index will normally imply a change in proliferation rate or, in other words, a change in cell cycle time or distribution of cell cycle times. The labeling index gives the same type of information as the mitotic index but for some tissues, such as the bone marrow, labeling techniques are much more easily measurable.

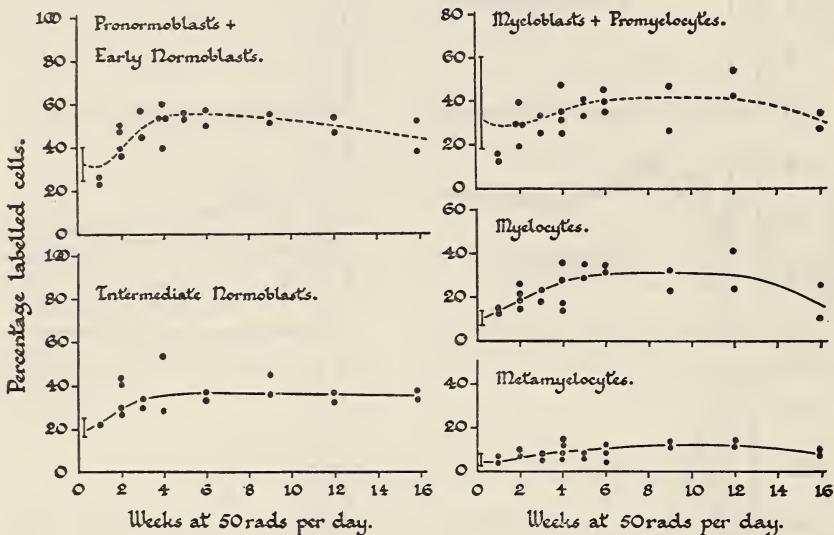
BONE MARROW

We find that the normal labeling index of nucleated cells of the bone marrow in our 8-week rats is about 15 percent. However, under



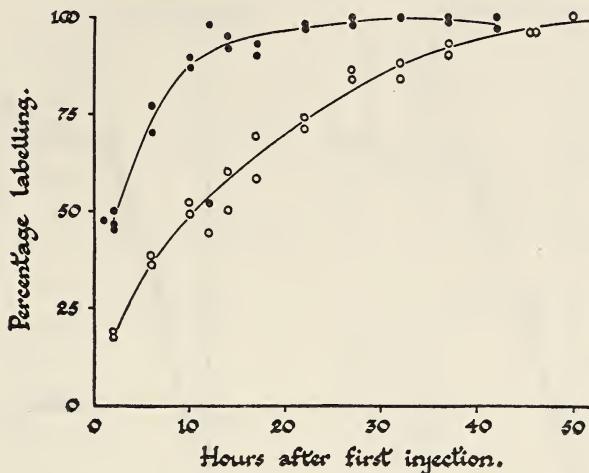
TEXT-FIGURE 6.—Recovery of peripheral blood counts following the removal of one third of the blood volume of normal rats and of rats irradiated continuously at 50 rads per day for 130 days before bleeding.

irradiation at 50 rads per day this increases over 3 to 4 weeks to a value of about 35 percent and is maintained at this value for 8 to 10 weeks before decreasing. An increase by a factor of more than 2 is also observed in the over-all mitotic index. As shown in text-figure 7, this increase in labeling index is demonstrated in both the red cell and white cell series, except for the earliest recognizable white cell forms (combined myeloblasts and promyelocytes) where the trend is not clear. There is evidence from this and other experiments that the red cell series can maintain the increase in labeling index longer than the white cell series.



TEXT-FIGURE 7.—Percentage of labeled femoral bone marrow cells, of different types, after the injection of tritiated thymidine during continuous irradiation at 50 rads per day.

Another demonstration of the change in proliferation pattern of the bone marrow under continuous irradiation is provided by studies with repeated injections of tritiated thymidine, with repeated sampling. Text-figure 8 shows the change with time in percentage labeling of intermediate normoblasts during repeated injections of tritiated thymidine (every 4 or 6 hours), for control and continuously irradiated rats. In this experiment the dose rate was 84 rads per day, but we would expect the general findings to be similar at 50 rads per day. In a proliferating population of cells of one type, the time required for all the cells to become labeled will give the maximum cell cycle time in the population, after a correction for the length of the synthesis period. The interpretation of the results is much more complicated in a cell system such as the bone marrow, in which there is a sequence of cell types of different morphological characteristics, but the much more rapid rise to 100 percent labeling in the irradiated animals indicates a substantial change in cell proliferation pattern; it is difficult to say at the moment to what extent this implies a reduction in the maximum



TEXT-FIGURE 8.—Increase in percentage of labeled intermediate normoblasts during repeated injection of tritiated thymidine (every 4-6 hours) in normal rats and rats given 84 rads per day for 15 days. ● = 84 rads per day for 15 days, ○ = normal.

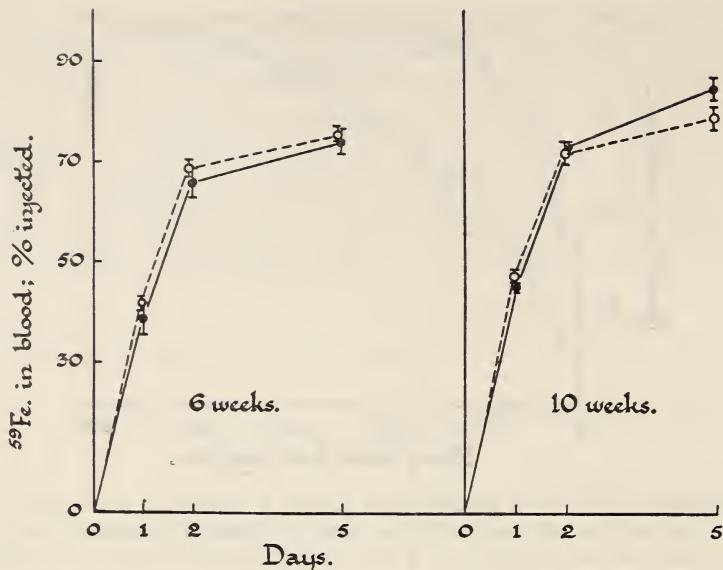
cell cycle time or a shortening of the residence time of a cell in the intermediate normoblast compartment.

A decrease in mean cell cycle time does not necessarily imply an increased output of mature cells. If the stem cell output remains the same the effect of a reduced cell cycle time in the proliferative compartment will be merely to reduce the residence time in that compartment, unless there is a greater number of divisions between stem cell and mature cell.

For the whole bone marrow population it is difficult to obtain information on stem cell capacity or gross output of mature cells, but relevant data can be obtained for the red cell system. Using radioactive iron techniques one can measure the rate of red cell production and text-figure 9, taken from the work of Dr. N. M. Blackett, indicates little difference in the rate of red cell production between normal rats and rats that have been continuously irradiated at 50 rads per day for 7 weeks and 11 weeks.

It is also possible, with a technique essentially the same as that of Hodgson (4), to make a determination of what is sometimes called the stem cell content, but what is actually the red cell repopulating ability of the marrow. The parameter measured is the ability of a given number of bone marrow cells to repopulate the erythropoietic system of a rat given 600 rads whole-body radiation. The repopulating ability is measured by radioactive iron incorporation in the recipient rat, 7 days after the marrow transplant. Marrow taken from rats given 50 rads per day for 7 or 11 weeks is found to be considerably less effective than marrow from normal rats, a transplantation of 50×10^6 nucleated cells from the marrow of continuously irradiated rats being only as effective as 20×10^6 cells from control rats.

If one can equate repopulating ability with stem cell content, it would appear that in the continuously irradiated red cell system there is a



TEXT-FIGURE 9.—Incorporation of Fe^{59} into newly formed circulating red cells of rats irradiated continuously at 50 rads per day for 6 and 10 weeks and for control animals of the same age. ●—● = 50 rads per day; ○---○ = controls.

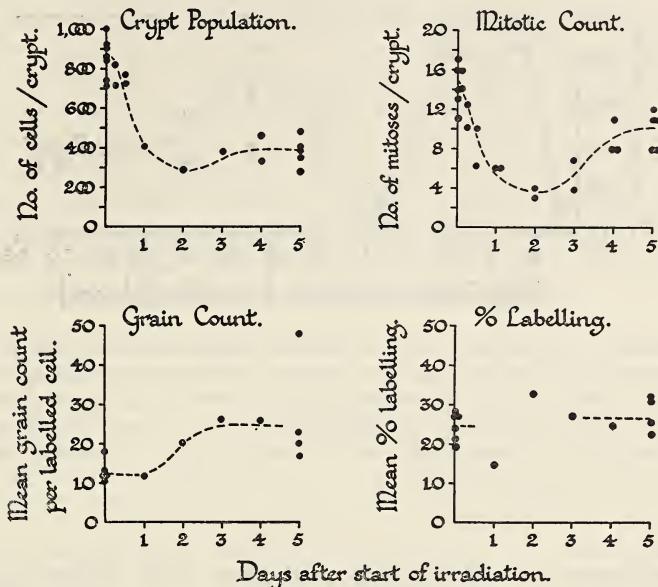
normal red cell output with a reduced stem cell content and also a reduced bone marrow precursor population (text-fig. 5). This would suggest a greater number of cell divisions between stem cell and mature cell but, since the proliferation rate is increased, the maturation time of the cell would not necessarily be increased—it could even be decreased.

A change in the number of divisions in the precursor line of the red cell might lead to a change in hemoglobin content per cell, and this will have to be investigated.

There is obviously a great deal more work required for a fuller understanding of the change in the normal division-differentiation pattern occurring in the bone marrow under continuous irradiation. One important question is whether there is an extension of division beyond the normal stage of cell maturation at which it is completed in the normal animal. We have some evidence for this in our studies, but it is not yet conclusive.

THE GUT

In the gut, even at 415 rads per day continuous irradiation, we find that the pattern of cell proliferation is much less changed than in the bone marrow at 50 rads per day, once the steady state of cell population has been reached. The percentage labeling index is, in fact, only slightly higher than normal, as can be seen from the lower right hand curve of text-figure 10. The data are not very extensive but sufficient to show

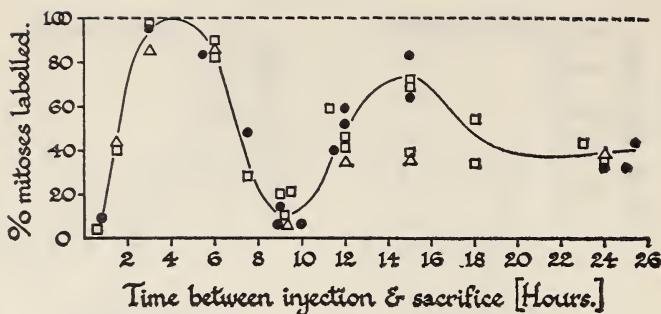


TEXT-FIGURE 10.—The response of the small intestine of rats exposed to 415 rads per day.

that there is no change in labeling index comparable to that in the bone marrow at 50 rads per day. Text-figure 10 also shows the change in the number of mitotic figures per crypt. There is a fall during the period of stabilization of the crypt count, but then a rise to a value 50 to 60 percent of normal, which indicates some increase in mitotic index, but much less than that observed in the bone marrow at 50 rads per day.

The rise in grain count of cells under continuous irradiation also shown in text-figure 10 will not be discussed here, except to say that it appears to be the result of a greater availability of tritiated thymidine in the irradiated animal.

Further information on a possible change in cell cycle time in the gut can be provided by the percentage labeled mitoses method (5). Determinations are made of the percentage of mitotic figures labeled at various times after a single injection of tritiated thymidine. The oscillations in the value of this parameter allow a direct determination to be made of cell cycle time. Data for the small intestine are shown in text-figure 11. Curves are given for control rats, for rats irradiated at 415 rads per day for 5 days, and for rats 2 days after removal from the unit following 5 days of exposure. The curves are essentially the same, which indicates no substantial change in cell cycle time from the control value of 10 to 11 hours. However, the period of the first oscillation of the percentage labeled mitoses curve is determined mainly by the cells of shortest cycle time present in the population, and the present results would not be inconsistent with an extension of cell proliferation in the crypt under continuous irradiation, and we are now investigating this.



TEXT-FIGURE 11.—Variation in the percentage of labeled mitotic figures, in the intestinal epithelium of rats following the injection of tritiated thymidine, in rats irradiated for 5 days at 415 rads per day, in rats allowed 2 days recovery after 5 days irradiation at 415 rads per day, and in control animals. August strain, male: ● = control animals; □ = after 5 days at 415 rads per day; △ = after 5 days at 415 rads per day + 2 days recovery.

DIFFERENCES IN RESPONSE OF GUT AND BONE MARROW

One would expect that the effect of continuous irradiation *alone* would be to increase cell cycle time. Continuous irradiation of cells *in vitro* should show whether this is so. Unfortunately, our own experiments on irradiation of lymphoma cell cultures are not yet complete.

A reduction in cell cycle time under continuous irradiation will indicate the operation of homeostatic mechanisms. The results with the bone marrow suggest that there is normally a considerable spread of cell cycle times but that, at 50 rads per day continuous irradiation, there is sufficient cell death to stimulate a narrowing-up of the distribution of cell cycle times accompanied possibly by an increase in number of divisions between stem cell and mature cell. The lack of any marked change in cell cycle time of the gut, even at 415 rads per day, may well be a reflection of the fact that the gut is dividing as fast as a mammalian cell can. Dr. D. Wimber has shown that the cell cycle time in cells of the tail bud of the embryo mouse, where one would expect very rapid cell division, is also about 11 hours, and the duration of the various phases of the cycle is similar to that of the small intestine in the growing rat. The present findings, of relatively little change in cell proliferation kinetics of the gut under continuous irradiation, may apply only to young animals. Recently Dr. S. Lesher, while working in our laboratory and using the method of percentage labeled mitoses, demonstrated a shortening in cell cycle time of 2 hours or so in the gut of animals exposed to periods of 15 weeks irradiation at 50 rads per day. He showed, however, that the cell cycle time is increased with age; perhaps the capacity to return to the faster rate is retained and can be manifested under stress.

What is the reason for the much greater tolerance of the gut to continuous irradiation, compared with the bone marrow? An attractive

theory is that the shorter the cell cycle the less the effect of a given dose rate. This would be true if there were essentially a wiping-out of damage at each cell division. This theory would imply, if the data on bone marrow proliferation have been interpreted correctly, that the continuously irradiated bone marrow should be more resistant than normal bone marrow: none of our experiments so far have indicated this.

However, there may be special factors operating in the gut, such as a changing concentration of chemical protectors (for instance, serotonin) or of a degree of anoxia, which would produce a change in radioresistance. Further experiments are needed to test these possibilities.

RESUMEN

Se presenta un estudio de la respuesta de varios tejidos, en ratas jóvenes, a la irradiación continua sobre una escala de dosificación. Se ha encontrado que los tejidos de renovación del cuerpo difieren grandemente en la dosificación que pueden tolerar. Por ejemplo, el epitelio del intestino delgado puede mantener su función y una población celular estable (un tanto más pequeña que la normal) a una dosificación tan alta como de 400 rads por día. La médula ósea puede mantener una población celular estable solamente a la dosis de 50 rads por día ó menores, y el testículo es mucho más sensible a la irradiación continua que la médula ósea. Los distintos tejidos también varían en el modo en que el modelo normal de proliferación celular cambia bajo la irradiación continua. A una dosificación de 50 rads por día la médula ósea muestra un considerable aumento en la tasa de proliferación, mientras que el epitelio del intestino delgado demuestra solamente un ligero aumento. Estudios hechos en el sistema eritropoyético bajo irradiación continua indican que, a pesar de una reducción en la celularidad de la médula ósea (incluyendo el contenido de "células stem"), la producción de células rojas difiere poco de lo normal. Esto sugiere un cambio radical en la cinética de la población celular, y esto se discute.

También se refieren estudios sobre la respuesta de tejidos bajo irradiación continua al sobrestímulo que demanda una proliferación incrementada. La conclusión es que la exposición continua no conduce necesariamente a una acumulación de daño que afecte la capacidad regenerativa de los tejidos de renovación, aún a dosis bastante altas.

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DISCUSSION

Upton: I think that Dr. Lamerton is to be complimented for his presentation which illustrates the value of the quantitative physical approach in physiology. I was interested in his speculation that the relative resistance of the crypt cell, as opposed to the marrow stem cell, may result from its shorter generation time and thus its higher cumulative dose between divisions. Such an explanation might help to account, in part at least, for the inability of the spermatogonium to withstand continued irradiation. I should like to inquire whether shortening of the villus in the small intestine was observed, since in the marrow the reserve compartment was decreased; did the villus decrease in length correspondingly?

Lamerton: With regard to the relationship between cell cycle time and sensitivity to continuous irradiation, we have done some preliminary work on two types of transplantable animal tumors with different cell cycle times. The one with the shortest cycle time is the more resistant, but we have to do more work before we can generalize. Concerning the length of the villus under continuous irradiation there is some shortening, but also a reduction in diameter, so that the cell population of the villus is reduced.

Effects of Radiation on the Synthesis of Nucleic Acids in Polytene Chromosomes^{1, 2}

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SUMMARY

Experiments were done using larvae of *Rhynchosciara angelae* (Diptera, Sciaridae) to study the effect of ionizing radiation on the synthesis of deoxyribonucleic acid (DNA). Since the cells of the salivary gland of these larvae do not divide, it is possible to study the effect of radiation on the synthesis of DNA in the absence of the complicated steps of the mitotic process. We gave to different groups of larvae 1250, 2500, 10,000, and 20,000 roentgens from a source of γ -rays, and analyzed the effect

of radiation on these chromosomes. For this we used autoradiography and tritiated thymidine as radioactive precursor to DNA. In cells of the salivary gland of *R. angelae* the radiation (even the 20,000 roentgen dose) seems to stimulate the synthesis of DNA, as shown by the many cells incorporating tritiated thymidine and also by the higher intensity of incorporation in chromosomes that received radiation as compared to nonirradiated controls. *Nat Cancer Inst Monogr* 14: 199-204, 1964.

SINCE ionizing radiation can induce malignant tumors and also can be used to cure them, it is very important to know better what is the effect of this physical agent on living cells. Until a few years ago there was a general belief that radiation would inhibit deoxyribonucleic acid (DNA) synthesis, but more recent data have shown that, depending on the dose, radiation may actually enhance this synthesis (1). It is well known that radiation is more active in dividing cells than in tissues where cell divisions are rare or absent. As our knowledge of the structure of the cell parts as well as of their functions is relatively poor, we have to look for convenient methods and materials to analyze this problem. One of the difficulties in the analysis of the effect of ionizing radiation on dividing cells is that in this process there are so many different interrelated reactions. For instance, it is very difficult to separate a direct from an indirect effect of radiation in any specific step in the chain of reactions that normally

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occur. The possibility of analyzing the effect of radiation in cells where the synthesis of DNA occurs without a subsequent cell division offered itself as an extremely convenient case. We used for this, the polytene chromosomes which are characteristics of the two-winged flies (Diptera). In these flies, at larval stage, the synthesis of DNA occurs in the nucleus of cells of several tissues, for a long period, without cell multiplication. In this material we have strong evidences in favor of the idea of a stimulating effect of radiation on the synthesis of DNA, even for such high doses as 20,000 r of γ -rays.

RHYNCHOSCIARA ANGELAE (DIPTERA, SCIARIDAE)

The material we used were larvae of *Rhynchosciara angelae*, a very favorable material for the study of chromosomal physiology as well as for the synthesis of nucleic acid in these nuclear elements. In these flies hundreds of larvae, all descendants of one female, live together for about 60 days and at any time these larvae, which have a synchronous development, have the same age and are of the same sex. They pupate together and after about 10 days practically all the adults emerge at the same time. In the larvae one can find very large polytene chromosomes in cells of several organs, which are particularly favorable for fine structure observation in the cells of the salivary gland. Using radioactive precursors of nucleic acids and protein (2-4), we could follow the synthesis of these chemical components of the polytene chromosomes in different organs and at different stages of larval development. In *R. angelae* the synthesis of DNA, although occurring at any time of larval development for individual nuclei, is an intermittent process. The length of time of nonsynthesis of DNA is variable, depending on the phase of larval development. At practically any time during the development of the larvae, if one injection of tritiated thymidine is given, and the autoradiographic method applied, the presence of chromosomes with different concentration of the radioactive precursor of DNA is always found. In certain cases one can see in adjacent cells that one cell shows a very high concentration of the radioactive precursor while the other cell does not show any incorporation. In the experiment done by Pavan *et al.* (5), 15 larvae of a homogeneous group were selected and separated in three smaller groups of 5 larvae each.

One of these groups of larvae was given a single injection of tritiated thymidine, in another group two injections were administered 30 minutes apart, and in the third group three injections were also given 30 minutes apart. The supposition was that the tritiated thymidine would be active inside the larvae as precursor of DNA for a period of 30 minutes, the first group of larvae would have it active for 30 minutes, the second for 60 minutes, and the third for about 90 minutes. The results obtained show that 70 percent of the cells of the larvae of the first group, about 90 percent in the second group, and over 99 percent in the cells of the third group were marked, that is, they showed incorporation of tritiated thymidine.

In addition to this experiment, we performed various similar ones, all of which concluded that the synthesis of DNA is always present in the salivary gland of *R. angelae*, although the synthesis in a single cell is an intermittent process. This intermittency is shown by the fact that the cells which do not incorporate the available tritiated thymidine, after one injection, will on longer exposure to the precursor finally show incorporation. The frequency with which different cycles of synthesis of DNA occur in a single cell of the salivary gland of *R. angelae* depends on the stage of larval development. The larvae in the experiment mentioned were near pupation, and at this phase of development the interval of non-incorporation of tritiated thymidine seems to be no more than 100 minutes. This allows us to conclude that in this phase of larval development of *R. angelae* a cycle of synthesis of DNA in the polytene chromosomes of salivary gland would duplicate only a portion of the strands present. Our preliminary data suggest that in the last week of larval life (before pupation) there are several cycles of DNA synthesis for each cell, and if the polytene chromosome would duplicate each time that DNA synthesis occurs, the polytene chromosome should be much larger than it is.

Other publications (2, 6-8) have shown that the quantity of DNA synthesized at any single band is variable when different tissues are compared or if the same tissue is observed at different phases of larval life. Another interesting result (to be published elsewhere) is related to the pattern of synthesis of DNA at each cycle. We have observed that the synthesis of DNA in the chromosomes follows a definite pattern: At the beginning the synthesis starts at certain bands, then passes to others, and still later more remote bands start to synthesize DNA. The pattern of incorporation of DNA in the polytene chromosome of the salivary gland of *R. angelae* seems similar at different stages of larval life, but the amount of DNA synthesized for each specific band is variable during larval development.

Using physical and chemical agents, we tried to interfere with the synthesis of DNA in these chromosomes. Our results with γ -rays follow. From a uniform group of female larvae, all descendants of a single female, we took six groups of 10 larvae each. To four of these six groups we gave, respectively, 1250, 2500, 10,000, and 20,000 r of γ -rays (cesium-137 source). The six groups were then injected with tritiated thymidine after which smears were made from the cells of the salivary gland and the slides were prepared by the autoradiographic method (Kodak Stripping film AR10). The two groups not receiving radiation from the cesium-137 source were taken as control. One of them had the same manipulation of the group which received 1250 r (the control did not receive the radiation) and the other had the manipulation of the group that received 20,000 r. This last group had to stay in a special vial for about 6 hours, the time necessary to get the 20,000 r. The second control was put in a similar vial and stayed there for the same time as the first control. The results are presented in table 1.

TABLE 1.—Effect of radiation on the synthesis of deoxyribonucleic acid in polytene chromosome of *R. angelae*

Groups of 5 larvae	Radiation dose (r)	Mean No. and percent of chromosome showing:			Total chromosomes analyzed	Minimum and maxi- mum percentage of marked chromosomes in the individual analyzed
		No incorporation	Low incorporation	High incorporation		
A	Control I	185 (48.05)	76 (19.74)	124 (32.20)	385	50.57, 60.76
	1250	47 (15.88)	48 (16.21)	201 (67.90)	296	69.13, 89.46
B	2500	91 (24.93)	87 (23.83)	187 (51.23)	365	71.42, 79.13
C	10,000	15 (6.94)	57 (26.38)	144 (66.66)	216	83.99, 96.63
D	20,000	48 (15.04)	88 (27.36)	183 (57.36)	319	65.21, 100.00
E	Control II	59 (22.69)	99 (38.07)	102 (39.23)	260	69.09, 82.00

As shown in table 1 chromosomes were classified as having no incorporation, low incorporation, and high incorporation. We planned to count the number of grains of silver in the emulsion so we could better classify the amount of tritiated thymidine absorbed by each chromosome, but this counting proved impracticable. The classification we used is not the best, but it is sufficient to give us a good idea of the effect of radiation in the incorporation of tritiated thymidine in polytene chromosomes of *R. angelae*. The first conclusion which may be derived from the results is that radiation—even as high as 20,000 r of γ -rays—does not inhibit DNA synthesis in these chromosomes. If we compare the results obtained in the different groups receiving different amounts of radiation, it looks as if the larvae which received radiation show in general not only a higher frequency of cells exhibiting incorporation but also a higher frequency of chromosomes showing more intense incorporation. If we analyze the last column of table 1 we see that at least one larva which received 20,000 r showed incorporation of tritiated thymidine in all cells of its salivary gland, a situation not found in larvae of other groups.

All the injections in this experiment were done in the first half hour after the irradiation and the different groups of larvae received 1250, 2500, 10,000, and 20,000 r in 22.5, 45, 180, and 360 minutes, respectively. It is possible that the radiation paralyzes some metabolic process inside the cell related to the DNA synthesis, and as soon as the radiation is stopped the process starts to function again. If this is so, several cells would start to incorporate simultaneously and this would mislead us into interpreting the effect as the stimulation of the synthesis by radiation. We do not have data to separate these two effects.

We have data on the effect of radiation in the synthesis of ribonucleic acid (RNA) in these chromosomes, and there our data also show that doses as high as 20,000 r have no marked effect in RNA synthesis. In addition we have data showing that internal radiation (emitted by H^3) does not have much effect in formation of puffs in polytene chromosomes (9). Larvae were injected with heavy doses of tritiated thymidine about 7 days before puff formation; when the larvae were killed, 7 days after receiving injections, the chromosomes exhibiting very high incorporation of tritiated thymidine showed puffs similar to the ones present in chromosomes which had no or a low degree of incorporation of the DNA precursor. During puff formation on polytene chromosomes the normally occurring syntheses of DNA, RNA, and proteins appear to be unaffected by the tritium radiation. We are now trying to analyze the effect of radiation on the synthesis of DNA in specific chromosome loci (bands), but as yet we have no positive results.

In conclusion, we may say that the effect of radiation on the synthesis of nucleic acids on living beings cannot explain in itself the obscured effects of radiation. To find what are the primary effects of radiation on the cells is still the challenge which we must meet, and we are obligated to gain the use of ionizing radiation as a tool for human welfare to win this battle.

RESUMEN

Se realizaron experimentos para estudiar el efecto de las radiaciones ionizantes sobre la síntesis de DNA, utilizando larvas de *R. angelae* (Diptera, Sciaridae). Como las células de las glándulas salivares de estas larvas no se dividen, es posible en ellas, estudiar el efecto de la radiación sobre la síntesis de DNA en ausencia de los pasos complicados del proceso mitótico. Expusimos diferentes grupos de larvas a 1.250, 2.500, 10.000 y 20.000 roentgens de una fuente de rayos gamma, y analizamos el efecto de la radiación sobre sus cromosomas. Usamos para ello el método de autoradiografía, y timidina tritiada como precursor radiactivo del DNA. En las células de las glándulas salivares de *R. angelae*, la radiación parece estimular la síntesis de DNA como lo muestra el mayor número de células que incorporan timidina tritiada, así como la mayor intensidad de incorporación en los cromosomas que recibieron radiación, en relación a sus controles.

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Radiation-Induced Cancer

The Role of Radiation on Experimental Leukemogenesis¹

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SUMMARY

It has been established that radiation induces lymphosarcomas in strain C57BL and other mice by an indirect mechanism, whereby tumors develop in nonirradiated thymus glands grafted to preirradiated hosts. It was subsequently shown that cell-free extracts from such radiation-induced lymphosarcomas, inoculated into nonirradiated newborn isologous mice, elicited identical tumors with significantly increased incidence and that the activity of the agent in the cell-free extracts increased after serial passage, which suggests that a virus or virus-like agent mediates the actual neoplastic induction in irradiated mice. Recent serial passage data are reported

in the present communication. In addition, evidence is presented to support the thesis that radiation produces three simultaneous effects, all of which are essential to the induction process: 1) injury to the normal sites of storage of the latent virus with concomitant release of virus; 2) injury to the thymus, followed by regeneration; 3) injury to the bone marrow, which in turn interferes with the regeneration of the irradiated thymus, producing a maturation arrest in which large numbers of highly immature lymphoid cells are made available for a sustained period for the oncogenic expression of the virus.—Nat Cancer Inst Monogr 14: 207-220, 1964.

IN ANY attempt to propose a pathogenetic mechanism for radiation-induced lymphosarcomas and lymphatic leukemias in mice, the following experimental observations must be considered (1-3). The dose-response relationship in strain C57BL exhibits an apparent "threshold" in the region of 200 r, below which the observed incidence is within the spontaneous range of 1 to 4 percent. An even more striking departure from expectation, if the mechanism of action were directly mutagenic, is the far greater tumor yield observed when a given dose is fractionated, with successive increments spaced several days apart (4, 5). The entire body must be irradiated; shielding of the spleen (6) or of active bone marrow (7) is dramatically protective, as is the intravenous injection of nonirradiated isologous marrow cells after systemic irradiation (7). The thymus is the locus of origin of virtually all of these induced

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Calli, Colombia, July 1-6, 1963.

neoplasms, and thymectomy prior to or even shortly after irradiation effectively prevents their development (8, 9). In grafting experiments, the genetically determined susceptibility of each strain is transmitted as an intrinsic property of the thymus (10). Tumor incidence closely reflects the endocrine environment of the host; the direction and magnitude of effect of any hormonal change with respect to tumor yield parallel its tropic effect on thymus growth (11). That an indirect induction mechanism is involved was established when tumors arising in non-irradiated thymus grafts transplanted to thymectomized irradiated hosts were identified immunogenetically as donor-derived (9, 12). This paradox was at least partially resolved when it was discovered that cell-free extracts of presumably viral nature, prepared from radiation-induced lymphomas of strain C3H (13) and C57BL (14) mice, were leukemogenic when inoculated into newborn isologous animals.

In this paper, my aims will be 1) to propose mechanisms of radiation action that are consistent with this complex set of observations; 2) to examine the available evidence supporting these proposals; and 3) to consider their relevance to the modes of action of other agents.

First, however, it seems desirable to present a progress report on the characterization to date of the radiation leukemia virus (RLV) of strain C57BL mice. Confirmation of the leukemogenicity of cell-free extracts from the radiation-induced lymphoid neoplasms of this strain has recently been reported by Latarjet and Duplan (15). Serial passage of the agent through some 15 tumor-induction generations in our laboratory continues to reveal a reduction in latent period, the current minimum being 50 to 60 days, and an increase in incidence, attaining 100 percent in several recent preparations, but with persistent variability from one preparation to the next (table 1). In the fifth transfer generation and again subsequently, serial passage preparations were inoculated into newborn strain AK mice and were found to accelerate the onset of the "spontaneous" leukemias of that strain. This acceleration phenomenon, originally discovered by Rudali *et al.* (16) after injection of newborn AK mice with extracts of AK leukemias, is not produced by RLV extracts prepared from fresh radiation-induced C57BL lymphomas. The serially passed RLV continues, however, to have a distinctly different host range than the corresponding AK (Gross) agent; the latter is inactive in strain C57BL and its F₁ hybrids and active in strain C3H, in which RLV is relatively inert. The host range of the agent extracted from RLV-accelerated leukemias of AK mice is that characteristic of the AK agent, rather than that of RLV. The host range of RLV remains much more restricted than that of the more potent Moloney leukemia virus (17).

Tissues from mice bearing leukemias induced by our serial passage preparations of RLV were examined by Dalton with the electron microscope; type C particles morphologically indistinguishable from those seen in mice inoculated with the Gross or Moloney viruses were demonstrated in their usual habitat, the megakaryocytes of the bone marrow (18). Many

attempts to establish an *in vitro* assay for RLV, with a variety of cell sources and culture techniques, have been frustrated by its lack of cytopathogenicity. The virus has been shown to withstand freezing and storage in liquid nitrogen, as well as fluorocarbon extraction. Efforts at its purification are now in progress, but are rendered discouragingly slow and imprecise by our dependence on *in vivo* assay. In summary, the evidence that the leukemogenic activity of cell-free extracts of C57BL radiation-induced leukemia is indeed attributable to a virus is now much more convincing, and some of the biological traits of the virus are now known, but its biochemical characterization must await further purification.

Although the virus must be present during much or all of postnatal life in nonirradiated strain C57BL mice, the mere presence of the virus obviously is a necessary but not a sufficient condition for leukemogenesis, and the other conditions requisite to its leukemogenic action seldom occur naturally in this low-leukemia strain. What does ionizing radiation do to modify the host-virus relationship, setting in train a sequence of events leading to leukemia development? We can immediately dismiss the possibility of direct radiation-induced change in the virus, since the effective dose range for killing or mutation of viruses is 1000-fold or higher than the radiation doses that consistently yield an incidence of 80 to 100 percent in this strain. Thus, we conclude that the leukemogenic effects of radiation are exerted on the tissues of the host and that any alteration in behavior ("activation") of the virus is secondary to these tissue changes. After consideration of the available evidence, I propose the thesis that radiation produces 3 simultaneous tissue effects, all of which are essential to leukemogenesis: 1) injury to the normal sites of storage of the latent virus, with concomitant release of virus; 2) injury to the thymus, followed by a stimulus to regeneration; and 3) injury to the bone marrow, which in turn interferes with regeneration of the irradiated thymus, producing a maturation arrest characterized by the presence of large numbers of immature thymic lymphoid cells.

Injury to the thymus and the bone marrow and the effect of bone marrow injury on thymic regeneration are firmly established by direct evidence that has been adequately detailed previously (5). The first effect, release of virus from its nononcogenic sites of replication by radiation-induced tissue injury, is rather speculative and is supported to date largely by indirect evidence. This evidence must be considered in relation to the acceleration phenomenon in the spontaneously susceptible strain AK. Cell-free filtrates prepared from whole embryos of strain AK were reported by Gross (19) to exhibit leukemogenic activity. Therefore, it might be inferred that the virus is present during early life in at least some of the tissues of the body. Yet, the injection into newborn AK mice of virus from exogenous sources (AK lymphoma filtrates) was shown by Rudali *et al.* (16) to accelerate the onset of lymphoma development by as much as 3 months. It is difficult to account for this effect of additional virus unless one postulates that the congenitally acquired virus already present is

TABLE 1.—Leukemia incidence in selected passage generations of radiation leukemia virus (RLV)

Passage No.	Sub-passage	Source of RLV	Strain of donor	Strains injected	Number injected	Number* positive	Incidence (percentage)	Mean latent period (days)
Original	—	Early X-ray-induced lymphoma	C57BL	C57BL	15	2	13	388
3	—	Passage 2	ABL	C57BL ABL† C57BL	11 8 12	3 2 4	27	282
5	—	" 4	C57BL	ABL + BLA‡ BALB/c	13 6	6 2	33	585
7	K ₂	" 6	AK	AK C57BL C3H BLKS	13 6 5 5	10(12) 0 1 1	46 77 — 20	128
C	" 6	C57BL	AK C57BL ABL + BLA	12 14 10 6	9(10) 75(83) 71 32	157 83 32 217	91 131 111 —	

10	D	"	9	AK	C57BL BALB/c	8	1	12	—
				BLK	BLA	10	0	—	—
				AK	BLA	11	0	—	90
				C57BL	BLK	18	2(14)	11(78)	218
				BLA	BLA	15	9	67	21
				C57BL	BLK	6	0	—	—
				BLA	BLK	12	0	—	—
				C57BL	BLA	17	17	100	136
				BLA	BLA	12	5	42	223
				AK	AK	15	9(13)	60(87)	143
				BLK	BLK	7	2	29	226
				C57BL	C57BL	21	13	62	172
				BLA	BLK	5	1	20	190
				C57BL	C57BL	20	16	80	122
				AK	AK	8	7(7)	88(88)	130
				C57BL	C57BL	10	8	80	261
				AK	AK	5	3(5)	60(100)	131
				C57BL	C57BL	20	14	70	156
				AK	AK	10	0(4)	—(40)	—

*Represents the number of mice dying from leukemia, except for the AK mice, where it is the number of mice dying from leukemia before reaching the age of 170 days, i.e., the number of mice with accelerated disease. The number in parentheses is the total number of AK leukemia deaths.

^tABL = (BALB/c ♀ × C57BL ♂)F₁ hybrid.

^tBLA = reciprocal cross.

[§]BLK = (C57BL ♀ × AK ♂)F₁ hybrid.

segregated for the first few months of life in cells or tissues, such as the gonads (20) brain and spleen (21), which are refractory to its oncogenic action. This is supported by studies of the accelerating activity of cell-free filtrates prepared from the nontumorous thymuses of AK mice of different ages. There was little or no accelerating activity in the thymuses of mice 1 to 60 days of age, whereas definite activity was noted at 3 months of age and thereafter (22, 23). The correlation between the degree of acceleration obtainable (about 3 months) and the age at which accelerating activity first appears suggests that AK mice may fail to develop lymphomas at an earlier age because the virus is not present in their thymuses until some 3 months after birth.

Let us turn now to the indirect evidence which we interpret as indicating that radiation, through injury to tissues in which virus is "stored," releases the virus for possible leukemogenic action in the thymus. First, we may note the long-established paradox that several doses of radiation, spaced several days apart, are far more effective than the same total dose given either as a single exposure or at daily intervals (4). Our recent studies on the interaction between virus injection and irradiation may have provided the key to understanding this phenomenon. Striking synergism between virus and radiation is observed when cell-free extracts from our serial passage material are injected into young adult C57BL mice 1 week prior to irradiation, given either as a single exposure or as spaced fractions (24). An enhanced response of lesser magnitude is also noted when virus is injected after, rather than before, irradiation. The *interval* between injection and irradiation probably is important, consistent with Rauscher's (25) recent observation that another leukemia virus, after injection, undergoes an "eclipse" for several days, to reappear in the plasma again at about 7 days. It is an attractive hypothesis that each fractionated, spaced irradiation, by releasing endogenous virus, is in effect a kind of virus "injection," which can then interact with the next fractionated exposure to augment the leukemogenic yield. The same mechanism may also explain two other phenomena: 1) the development of lymphomas in nonirradiated thymus implants grafted into preirradiated hosts (10), and 2) the synergistic effect of thigh-shielded irradiations given 1 to 4 weeks before one or more doses of whole-body irradiation (26). More direct evidence, though limited, includes our recent preliminary observation that injection of pre-irradiated bone marrow cell suspensions into irradiated C57BL mice accelerates the onset and may also slightly increase the incidence of these leukemias (27), whereas similarly timed injections of normal bone marrow cell suspensions effectively protect irradiated recipients (9). Mathé and Bernard (28) have reported fragmentary evidence of leukemogenic activity in the plasma of irradiated C57BL mice, and Berenblum and Trainin (29) have noted enhancing activity in tissues from irradiated mice. More quantitative direct evidence is needed to support this point.

Unlike strain AK, mice of strain C57BL, though highly susceptible to thymic lymphoma induction by irradiation, are refractory to the spontaneous disease. Moreover, the susceptibility of nonirradiated C57BL

mice to the leukemogenic action of injected virus rapidly decreases within the first 2 to 3 weeks after birth. Morphologic studies reveal that the architecture of the newborn thymus is remarkable in that the outer third or half of the cortex is virtually a "pure culture" of large, immature cells. As differentiation proceeds during the first 10 days of life, this layer dwindles to a layer only one or two cells thick, and by 30 days it has disappeared (30). These observations have now been confirmed with more quantitative techniques in strain C3H thymuses by Axelrad and Van der Gaag (31). In contrast, in the hyperplastic thymuses of strain AK mice, shown by Arnesen (32) and later by Metcalf (33) to be associated with hypoadrenocorticism, an abnormally high proportion of such large, immature lymphoid cells persists into young adult life. Thus there appears to be a good correlation between the presence and persistence of an abundance of these cells and susceptibility to either the "spontaneous" (endogenous) or the exogenous virus-induced disease, which probably reflects a specific requirement by the virus for substrate cells in a particular stage of lymphoid differentiation (30).

Irradiation of young adult C57BL mice, in which these immature thymic lymphoid cells have virtually disappeared, produces thymic injury, with necrosis of adult cortical lymphoid cells, followed by regeneration from stem cell precursors. When the bone marrow (or spleen) is shielded during such irradiation, thymic regeneration and lymphoid cell maturation proceed more rapidly, with the concomitant result that the immature lymphoid cells are present in abundance only briefly. When whole-body irradiation is administered, the injury sustained by the thymus is the same, but regeneration is greatly delayed, apparently due to a maturation arrest in which large numbers of immature, mitotically active lymphoid cells persist for many days (5). Thus, whole-body irradiation, by simultaneously injuring both the thymus and bone marrow, appears to rekindle thymic susceptibility to the leukemogenic action of virus by recapitulating the newborn condition of the thymus with respect to its population of immature lymphoid cells. That the injury sustained by the thymus need not be specific and that it is primarily what happens during regeneration which is important are clearly brought out by studies of the histologic changes in nonirradiated thymic grafts implanted into thigh-shielded *versus* whole-body irradiated hosts (34, 35). The thymus graft, temporarily deprived of its blood supply, undergoes near-total necrosis, with persistence in most instances of a thin rim of large reticular cells immediately beneath the capsule. When the host femoral marrow has been shielded, the thymic graft quickly regenerates, presumably from the surviving subcapsular cells in most instances, and again the maturation stage in which immature lymphoid cells are present in abundance is relatively brief, whereas regeneration is delayed and often incomplete in thymus grafts residing in whole-body irradiated hosts.

The nature of the stimulus to regeneration of the injured thymus is unknown, but it is clearly influenced by the same endocrine factors to which thymic growth and involution are normally so sensitive (36).

Thus the adrenocortical steroids and testosterone, which are strongly thymolytic, powerfully inhibit lymphoma development in irradiated C57BL mice, whereas adrenalectomy and orchiection increase the yield of these tumors, the increase being greater in the former case than in the latter, in keeping with their relative thymotropic potencies. The radiation-involved thymus is unable to respond to the tropic stimulus of adrenalectomy unless the bone marrow is shielded (11). The picture emerging is that of the sustained disturbance by irradiation of a complex and dynamic tissue equilibrium in which endocrine balance, bone marrow radiation injury, and availability of free virus appear to be vectors. Thymic susceptibility to the leukemogenic action of the virus is correlated with, and may be measured by, the disparity between the *expected* thymic weight in a mouse of a given strain, age, sex, and hormonal state and the *actual* weight to which the thymus is reduced by radiation injury, from which it is prevented from recovering by concomitant bone marrow injury.

Finally, it may be of interest to consider, in the context of this complex system, what is known or may be surmised regarding the action of certain other agents:

1. The adrenocortical steroids and testosterone, which are powerful thymolytic agents, fail to induce lymphomas in C57BL mice, most probably because they simultaneously suppress the stimulus for thymic regeneration (11). There is no evidence that they influence the distribution or replication of the virus.

2. Urethan potentiates the leukemogenic action of X rays, if given to adult mice with or after, but not before, irradiation (37, 38). Although it produces very few thymic lymphomas in unirradiated adult mice (37), it is an effective and complete leukemogen when administered in appropriate doses to newborn mice (39, 40). Recent studies in our laboratory (41) indicate that the susceptibility of bone marrow to the impairment of its thymus-regenerating capacity by urethan (42) is dose-dependent and undergoes an abrupt decrease during the pubertal period. In adult mice, urethan causes thymic injury similar to, but of briefer duration than, that caused by X rays (37). In the light of the leukemogenic synergism just described between virus injection and subsequent irradiation, the failure of prior urethan injection to synergize with irradiation suggests that urethan lacks the capacity to "inject" (release endogenous) virus in adult mice, whereas its leukemogenicity in newborn mice indicates that at this age urethan can elicit the same triad of effects (virus release, thymic injury, and bone marrow injury) as irradiation. Current experiments still in progress (41) indicate a distinct synergistic response to injection of RLV followed by urethan.

3. Lymphoid tumors induced by certain carcinogenic hydrocarbons have recently been shown to yield cell-free extracts, again presumably viral, with leukemogenic activity (43). Although the hydrocarbons are known to cause thymus involution, there is need for more detailed studies of thymic and bone marrow changes as a function of dose and chemical configuration

in mice of different ages and strains before it can be determined whether the pattern of hydrocarbon leukemogenesis is consistent with that proposed here for radiation.

RESUMEN

Se ha establecido que la radiación induce linfosarcomas en la cepa C57BL y en otros ratones por un mecanismo indirecto por el cual los tumores se desarrollan en las glándulas tímicas no-irradiadas injertadas a huespedes pre-irradiados. Se ha mostrado subsecuentemente que los extractos libres de células de tales linfosarcomas inducidos por radiación, inoculados a ratones isólogos recién nacidos no-irradiados, provocaban idénticos tumores con un aumento significante de su incidencia, y que la actividad del agente en los extractos libres de células aumentaba después de pasaje seriado, sugiriendo que un virus ó agente viroide actúa como intermediario en la inducción neoplásica en los ratones irradiados.

Los resultados sobre recientes pasajes seriados se refieren en la presente comunicación. Además, se presenta prueba en apoyo de la tesis que la radiación produce tres efectos simultáneos, todos los cuales son esenciales para el proceso inductivo: 1) lesión de los sitios normales de almacenamiento del virus latente con liberación concomitante del virus; 2) lesión del timo, seguida por regeneración; 3) lesión de la médula ósea, que a su vez interfiere con la regeneración del timo irradiado, produciendo una detención de la maduración en el que gran número de células linfoides altamente inmaduras son puestas a disposición por un sostenido período para la expresión oncogénica del virus.

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DISCUSSION

Rogers: I would first like to make a comment in relation to urethan, as mentioned by Dr. Mori-Chavez (this volume). Urethan is a very strange molecule—it is an anesthetic, a mitotic poison, and causes lung tumors and a variety of other tumors. It is also a potentiator of virus infections, such as influenza, through its effect of destroying to a degree the lymphatic system, and in addition makes certain foul-tasting molecules tasteless when chemically joined as a terminal group. With the exception of the mitotic poisoning effect and the effect on the immune mechanism (lymphocyte poisoning), these effects are independent of each other.

There is a point that has always interested me in relation to leukemia and virus-induced lymphoma. The virus might be conceivably acting as an antigen. For example, if you give animals whole-body irradiation and then give them, instead of a virus, multiple exposures to an antigen such as typhoid vaccine, to what extent might you accelerate the occurrence of lymphoma in time?

Kaplan: I cannot answer that question.

Cristoffanini:* Dr. Kaplan, you stated that normal marrow, when injected into irradiated mice, will protect them from leukemia, but the injection of irradiated marrow will not protect but *enhance* the development of leukemia. I wonder if you have tried sonication of those cells to find out if this is a matter of putting in altered living cells or of releasing extra virus out of damaged bone marrow cells?

Kaplan: We have not tried sonication. Some years ago we tried to find out whether the effect of the bone marrow in influencing thymus regeneration was a cellular or a humoral effect, by cell fractionation by differential centrifugation. The

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activity was not in any of the cytoplasmic fractions, but resided in the nuclear fraction. However, we could not exclude the possibility that the intact cells were involved in the response. About 5 years ago, Dr. Irwin Berman and I put bone marrow into diffusion chambers in the abdominal cavity of irradiated test animals, and, of course, compared it with saline in diffusion chambers. In two successive experiments we had distinctly greater regeneration of the thymus in animals bearing diffusion chambers containing normal active bone marrow than in other instances. We made one very serious error at that point—we did a third, a fourth, and a fifth experiment! They did not work. We have rather given up that approach, but maybe we ought to go back to it. It still is not clear what this mechanism is. It seems to me very likely that it is a cellular phenomenon rather than a remote humoral phenomenon.

Mole: I would like to present three difficulties, which make me wonder whether the weight of the evidence supports the conclusions. The first, if I can put it quite unfairly, is that if you define a disease as starting in the thymus, what else could thymectomy do but stop it happening? In recent years people have found that thymectomy, especially of young mice, can produce general effects on the host. Now, as I understand it, you believe the effect of thymectomy in relation to radiation-induced leukemia is a specific removal of the thymus. When C57BL mice were given a standard course of radiation, the age at which they died of leukemia was, let me say, 200 days, in round numbers. Some animals will die without leukemia later in life, in about 500 days. When the mice were thymectomized those that did not die from leukemia died at about 300+ days, and those that did get leukemia died at about 450 days (H. S. Kaplan, J Nat Cancer Inst 11: 83-90, 1950). So that, when one is comparing the over-all incidence of leukemia, the occurrence of leukemia in thymectomized animals is being diluted by the animals that died earlier without leukemia. If the effect of thymectomy is at least partly on the host and has the effect of delaying the development of leukemia, perhaps you are drawing conclusions that are not completely correct.

The second difficulty is this: Although one can develop the cell-free extract by passage so that it becomes exceedingly efficacious in its action, does this necessarily prove that every initial production of leukemia is due to this agent? As I understand it, only in a relatively small proportion of primary leukemias can the presence of the agent be demonstrated.

The third difficulty stems from one of the facts that you mentioned at the beginning. There is a sharply sigmoid relation between dose of radiation and the induction of leukemias. I think that you have used just the right level of radiation to produce the experimental results. But, when you are working on the really steep part of the dose-response curve and you change the incidence of leukemia from 80 to 20 percent, say, you are really modifying the efficiency of the radiation by only a small proportion. This is what worries me about all the experiments on the effect of endocrines, for instance, and cell-free extracts: The radiation level may be chosen so that this kind of result can be obtained, and the experimental modifications may be merely altering the efficiency of the radiation by a small fraction.

Kaplan: First of all you have referred to the question of age at death from leukemia. One of the arguments in favor of stressing thymic lymphomas is that one is apparently dealing with a single disease, lymphosarcoma (lymphatic leukemia). When you refer to death of thymectomized mice as averaged at 450 days, what disease are you talking about? Certainly not thymic lymphomas. Nonthymic leukemias (if you want to call them that) do occur, but, as Dr. Upton mentioned, they are miscellaneous. They include reticulum cell sarcomas, myeloid leukemias, and plasma cell tumors. If it were possible to recognize with precision only those lymphocytic lymphosarcomas that are histologically of the same morphology as the thymus tumors, there would be the basis for a valid criticism. However, the numbers of these are extremely small.

Your second point is a very important one. I think that the difficulty here is almost entirely due to lack of sensitivity of the assay. We have no *in vitro* assay. The only

in vivo assay we have had until now has been the induction of tumors. In recent months we have tried two other approaches: One is the acceleration phenomenon in the AK strain. But, as I mentioned, the C57 agent freshly extracted from these tumors, unlike the serially passaged agent, did not accelerate the onset of the AK disease. Now we are given some new hope by the synergistic response which I described today. We think that we may be able to detect small amounts of virus in our freshly induced thymic tumors by adding a small amount of radiation to the test animals injected with fresh cell-free extracts.

I think your third point about experimental design is not correct. You suggest that there are radiation response levels at which, for example, we could not demonstrate synergism or inhibition. This is simply not true. It does not matter whether we start off with a 20 or 100 percent leukemia-inducing dose of radiation, if we use appropriate doses of testosterone or hydrocortisone we can suppress the tumor incidence to any level. The same is true for all the other endocrine influences that we have examined so far. Whether this pertains to the virus I cannot say now, because we do not have enough information. But we know that the virus synergistic response is observed in at least two different radiation schedules: either with fractionated doses which alone would yield about a 40 percent incidence, or single doses of 300 r, with which we would expect the radiation controls to develop only about a 10 percent yield.

It is a truism to say that experiments should be devised in such a way as to demonstrate the effect. It seems to me that there is no point in doing the experiment unless you set it up so that it will show the effect, and if you set it up in a way that does not show the effect, that does not detract from the fact that the effect might exist.

Upton: I would like to inquire about two matters. First of all, I think you demonstrated evidence suggesting that the effects of radiation do indeed involve the liberation of virus and that one can graft into the irradiated animal a nonirradiated thymus that then falls prey to the released virus. I wonder, therefore, if successive exposures at weekly intervals result in successive increments of virus and ever greater levels of virus in the blood stream. Or perhaps the successive exposures may have an added effect, namely, progressively increasing damage on the marrow, which somehow has to help the implemented thymus to restore itself.

Kaplan: It would be my guess that the basic reason why successive doses are far more effective than the individual dose is the successive *in vivo* injection of virus, and not the effect on bone marrow. I have two reasons for saying so. One is that in the work we reported some years ago, in which we followed the patterns of thymic injury and regeneration after single doses of radiation, after successive daily fractions, and after spaced fractions, we were unable to detect any difference in pattern of injury or repair of the thymus in thigh-shielded or unshielded animals. That is, the extent of bone marrow injury seemed to be just as great after one dose as it was after multiple doses, in terms of interference with thymic regeneration. Frankly, I was puzzled about this because I had hoped that this study would reveal the reason for the need for multiple-spaced irradiation, and it did not. Now the other side of the argument is very tenuous; it has to do with some evidence in the recent paper by Latarjet and Duplan. They found that, although C57BL mice given a single injection of virus at about 1 month of age were refractory to leukemia development, when they gave three injections 1 week apart, there were as many leukemias as in newborn mice given a single dose. This is based on small numbers of mice and it needs to be confirmed, but it supports the view that multiple doses of virus are perhaps important.

Upton: Have you encountered myeloid leukemia in your intact or thymectomized animals?

Kaplan: This will, perhaps, bring frowns from the pathologists in the audience. Long ago we found that the development of radiation-induced thymic lymphomas is pretty well completed by about 360 days of age. Since it seemed preferable to concentrate on one variety of leukemia, we have made it a policy to kill the mice and terminate the experiments at about 400 to 450 days of age. For this reason I have not looked systematically for the myeloid leukemias.

Leblond: One of the points in your paper was the finding by Dalton of virus particles in megakaryocytes. Have you looked for virus particles in large lymphocytes in the thymus where you would expect them to play a role? Also, have you made counts of these large lymphocytes?

Kaplan: I cannot speak for Dr. Dalton, but I know that Dr. William Carnes has made repeated attempts to see particles in both the freshly induced thymic lymphomas and in thymuses harvested at various intervals after radiation, before the obvious appearance of tumors, and uniformly he has failed. To my knowledge, this has been true also in most instances for the Gross and Moloney virus-induced tumors.

As you know, Axelrad has done very careful counts in strain C3H mice, in which the decrease in susceptibility to the virus follows the same time course as it does in our animals. He was much more precise in quantitative measurements than we have tried to be. I think that it is perhaps not the absolute number of the large immature cells that may be important. If I can speculate on this, perhaps Dr. Luria will comment. If these cells constitute specific substrate for the oncogenic action of the virus, then it would seem that, unless one employed virus preparations of very high potency, virus emerging from one such cell would need to find another similar cell very close by in order to have a reasonable probability of continuing to propagate itself in that substrate. Thus, it is not so much the absolute number of large, immature lymphoid cells, but rather the probability that near a large cell there will be another large, immature cell instead of a cell surrounded by 10 small lymphocytes which are not susceptible.

Luria: I would find equally appealing, as an explanation of the role of the large lymphocytes, the idea that they may be a class of cells peculiarly adapted to respond to virus in an extreme morphogenetic way. I would not be surprised if a search for viral particles in tumor cells would actually fail if, for example, the cells which respond by becoming tumorous cells may be those that failed to produce mature virus, as with polyoma. I like the interpretation given by Kaplan, that the artificially created availability of cells may control the morphogenetic response to an agent whether it is a virus, a product of another gene from the same cell, or some substance coming from the outside. If there is a population of cells with a certain propensity to a given morphogenetic response, the absolute number of cells may reflect a need for proximity or may be simply a matter of probability that at least one cell responds in the critical way.

Upton: Recently Furth and his associates at the Toronto meeting of the American Association for Cancer Research reported tremendous numbers of Gross virus-like particles in thymuses of neonatally infected rats. The neonatally infected rats respond to the Gross passaged virus by the development of a big thymus tumor, a thymus lymphosarcoma. Furth and his associates studied these tumors by electron microscopy and found, at least in some of them, tremendous numbers of virus particles in the tumor cells (Okano, Kunii, and Furth, *Cancer Res* 23: 1169, 1963).

Luria: I would like to recall what I said earlier about the Rous sarcoma because I think it is very important to avoid mistaking the presence of viral particles with the role of the agent. For 50 years the Rous sarcoma has been known to be a virus-induced tumor. Yet, the Rous virus, according to Rubin, does not produce any particles unless there is another virus present.

Comparative Aspects of Carcinogenesis by Ionizing Radiation¹

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SUMMARY

Ionizing radiations appear carcinogenic to all mammalian species studied thus far. Species vary widely, however, in the probability of induced neoplasms of any given organ and cell type, and within a species there are variations in susceptibility according to strain, sex, age at irradiation, physiologic condition, and other factors. The relation between radiation dose and probability of neoplasia is complex but suggests, in general, that the neoplastic transformation involves a multistage process rather than a single, one-hit type of alteration. Radiation of low linear energy transfer (LET) is generally more effective when administered at a high intensity than at a low intensity; high-LET radiation is, in general, relatively less dose-rate-dependent and more effective than low-LET radiation. Many of the carcinogenic effects of radiation

are reproduced by radiomimetic chemicals, especially those known to have mutagenic and chromosome-breaking potency; however, the combined effects of radiation and chemicals are not necessarily additive, which suggests that the agents may differ in mode of action. The evolution of neoplasia, furthermore, appears in many instances to require interaction of cancer-forming cells with extrinsic cancer-promoting factors in their environment, and in some cases the cancer-forming cells themselves need not be irradiated. Hence, the significance of mutagenic effects in carcinogenesis is not clear. In the pathogenesis of certain radiation-induced neoplasms, oncogenic viruses appear to be involved, the origin and role of which remain to be defined.—*Nat Cancer Inst Monogr* 14: 221-242, 1964.

DURING the half century since the carcinogenic action of ionizing radiation was first recognized, radiogenic neoplasms have been recorded in many species of laboratory animals and in man (1-3). The data suggest that all kinds of ionizing radiation can induce neoplasia and that virtually any type of neoplasm may be elicited, depending on the conditions of irradiation and of the exposed individual. Few attempts have been made, however, to analyze comparatively oncogenic effects in various species, nor has the carcinogenic action of radiation been compared systematically with that of other agents. Possible interactions between radiation and other oncogenic agents also remain largely unexplored.

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

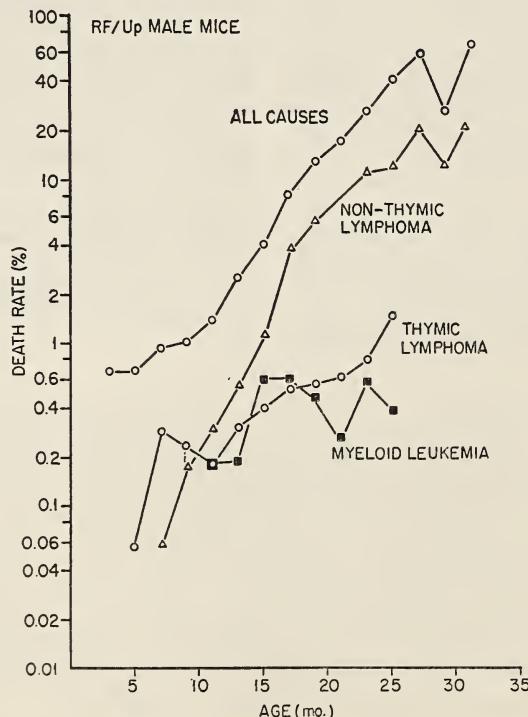
The following survey deals with some comparative aspects of radiation carcinogenesis from observations on animal and human populations.

LEUKEMIA

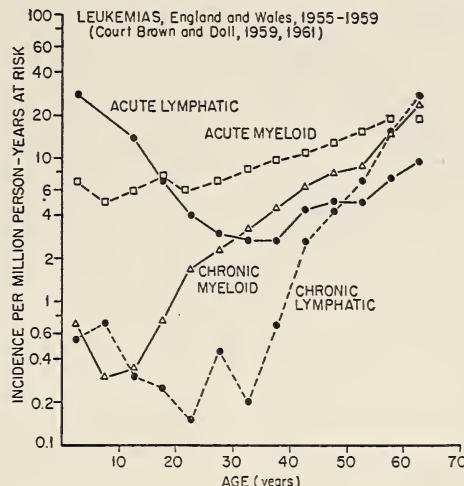
Variations in Hematologic Type With Species, Sex, and Age

The leukemogenic effects of radiation in humans and laboratory animals depend on the type of leukemia in question. In the mouse (text-fig. 1) as in man (text-fig. 2), the natural incidence of different forms of the disease varies markedly with age and with genetic or ethnic factors (4, 5). Irradiation tends to increase the age-specific death rate at all ages, if allowance is made for the induction period (text-figs. 3 and 4), but not all types of leukemia are affected equally, nor is susceptibility uniform with age and sex.

The data for the mouse indicate that susceptibility to induction of thymic lymphomas declines after involution of the thymus and is higher at virtually all ages in females than in males (text-fig. 5). Susceptibility to induction of myeloid leukemia, on the other hand, is relatively low in immature mice and higher at all ages in males than in females (text-fig. 6).



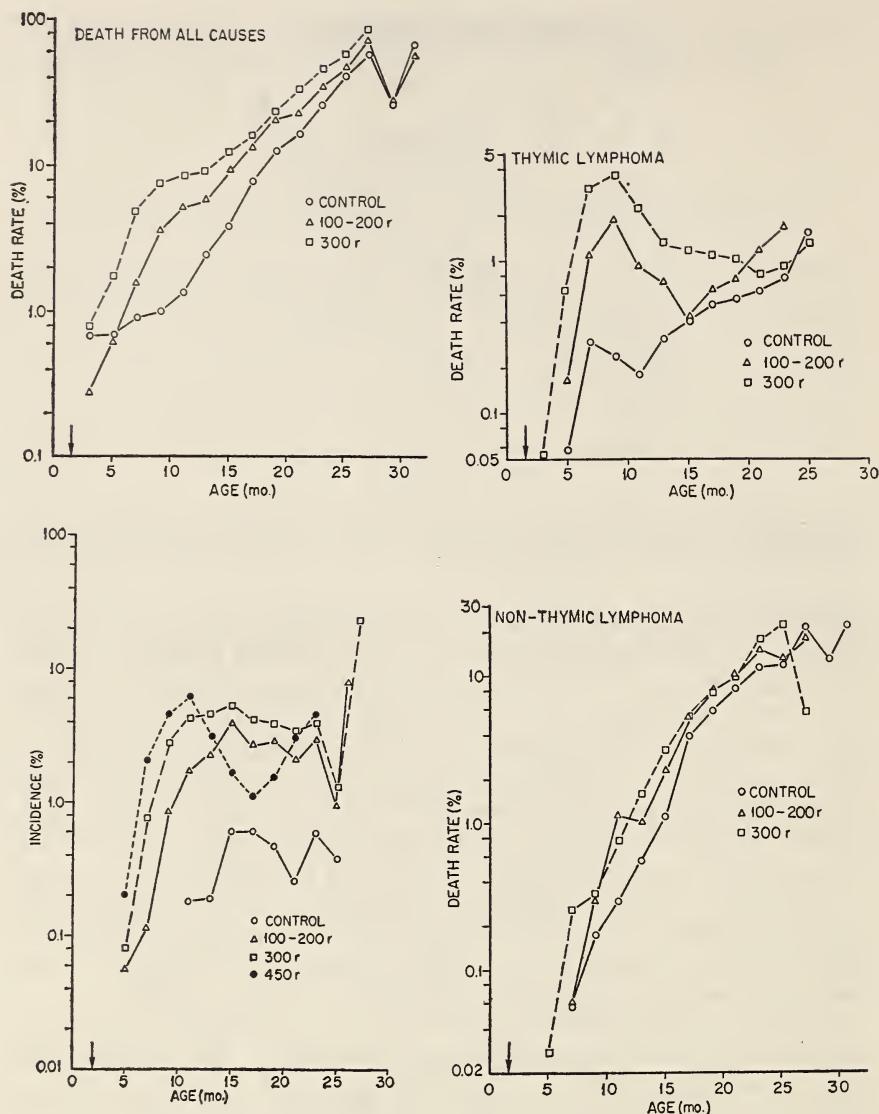
TEXT-FIGURE 1.—Age-specific death rate from leukemia in RF/Up male mice. Data from (78).



TEXT-FIGURE 2.—Age-specific incidence of leukemia in human population of England and Wales, 1955-59. Data from (80, 81).

Furthermore, mice irradiated at 2, 10, or 26 weeks of age show a biphasic elevation of the age-specific death rate from myeloid leukemia, and the rate remains elevated for the duration of the lifespan (text-fig. 3). Extrathymic lymphomas are not increased in frequency by irradiation in mice of most strains (6), although their age-specific incidence may be elevated slightly (text-fig. 3). In CBA mice, however, the incidence of nonthymic lymphomas may be greatly increased by 500 r of X radiation in fractionated exposures (7), and in thymectomized RF/Up mice the incidence of such growths may be similarly increased by a single exposure to 450 r (8), which indicates the influence of physiologic factors on susceptibility to this neoplasm. No type of murine leukemia or lymphoma shows a high incidence in immature mice which corresponds to the peak in frequency of human leukemia during childhood (text-figs. 1 and 2).

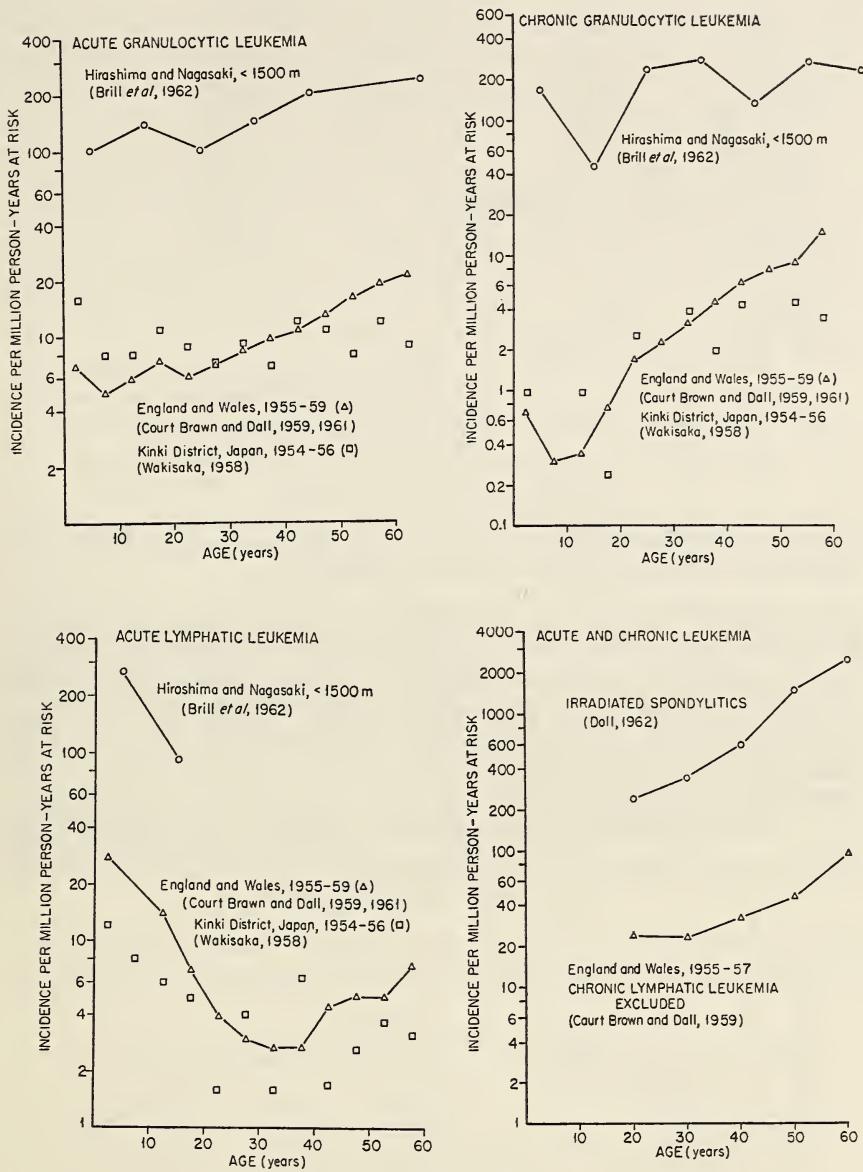
In man, the influence of age and sex on susceptibility to radiogenic leukemia is less well documented than in the mouse. The available data suggest that susceptibility in irradiated spondylitics increases with age at the time of irradiation (text-fig. 4) and is greater in females than in males (9), although too few females have been affected to provide quantitative data on sex differences. The greater increase in incidence among old, as opposed to young, patients is particularly striking in view of the higher spontaneous rate in old age; *i.e.*, to increase the incidence by the same factor, irradiation must induce many more cases in a population of elderly adults than in a population of young adults. In Japanese A-bomb survivors, susceptibility appears to have decreased with age at time of irradiation (text-fig. 4) and sex differences in susceptibility are not clear-cut, although they suggest that the males were more sensitive than females (10). In both populations, the incidence of acute leukemias and chronic myelogenous leukemia is increased, but there is no evidence of an



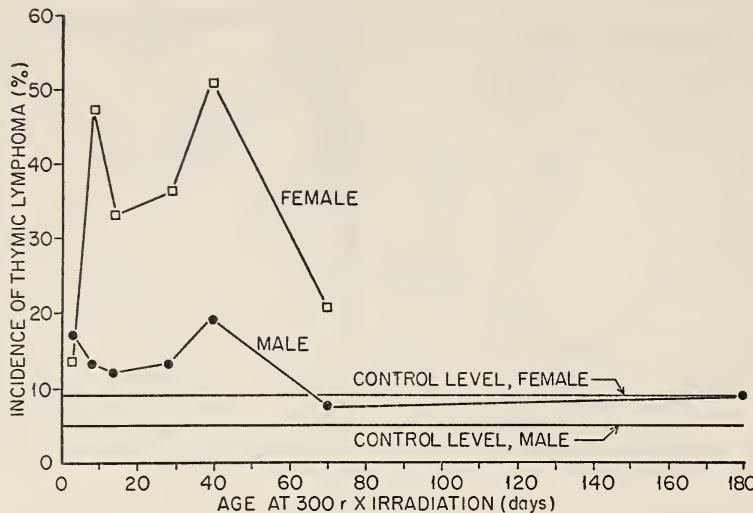
TEXT-FIGURE 3.—Age-specific death rate from leukemia in RF/Up male mice, as influenced by radiation. (Arrow denotes age at time of acute whole-body 250 kvp X irradiation.) Death from all causes; thymic lymphoma; myeloid leukemia; nonthymic lymphoma (from 78).

increase in the incidence of chronic lymphatic leukemia. The latter, however, is much rarer in nonirradiated Japanese than in Western populations (10).

The correspondence, if any, between leukemias of various types in the mouse and morphologically similar forms in man remains to be established. The myeloid leukemias of the mouse (6) ought to be classified as chronic



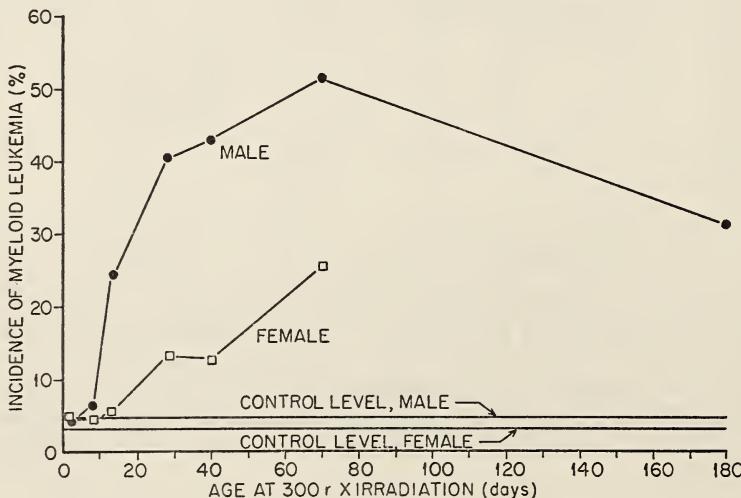
TEXT-FIGURE 4.—Age-specific incidence of leukemia in human populations as influenced by radiation and age at time of exposure. From (10, 32, 80, 81) and Doll, R., Brit J Radiol 35: 31-36, 1962. Acute granulocytic leukemia in A-bomb survivors, as compared with nonirradiated Japanese and British populations; chronic granulocytic leukemia in A-bomb survivors; acute lymphatic leukemia in A-bomb survivors; leukemia, all types excluding chronic lymphatic, in irradiated spondylitics.



TEXT-FIGURE 5.—Final cumulative incidence of thymic lymphomas in RF/Up mice, as influenced by age at time of irradiation. From (73).

on the basis of the degree of differentiation of the granulocytic series in the blood, marrow, and other tissues. But limited observations suggest that the duration of the disease is short, *i.e.*, a matter of days, which makes it more comparable to the acute form in man. Unlike any of the leukemias of man, however, spontaneously arising myeloid leukemias of the mouse affect only aging adults.

The thymic lymphomas of the mouse have no obvious human counterpart. To the extent that they appear at an earlier age than other murine leukemias, are composed of lymphoblast-like cells, and are inducible by



TEXT-FIGURE 6.—Final cumulative incidence of myeloid leukemia in RF/Up mice, as influenced by age at time of irradiation. From (73).

radiation early in life, they resemble acute lymphatic leukemia of man, but such a comparison is tenuous at best. They are not common in immature mice, if they occur at all before adult life. The earliest case I have encountered in over 10,000 necropsied mice was in a female dying at 11 weeks of age.

The nonthymic lymphomas of the mouse are a complex assortment of proliferative diseases (6, 7, 11). Predominant are lymphosarcomas and reticulum cell sarcomas of varying morphology and organ distribution; growths resembling Hodgkin's disease are not uncommon in certain strains. On morphologic grounds, it is not proper to compare these neoplasms with chronic lymphocytic leukemia and lymphomas of man, although they resemble the latter in age distribution and in failure to be readily inducible by irradiation.

From epidemiological studies of children exposed to diagnostic irradiation *in utero* (12), one might expect a significant increase in the leukemia incidence of mice exposed to X rays before birth. We have failed thus far, however, to induce leukemia by irradiation of the mouse fetus or embryo (table 1). Although the negative response of our mice may indicate that the radiation dosage was too high (10-1,000 times higher than the dose to the children in question), the absence of a murine disease comparable to acute leukemia of childhood complicates direct comparison between the two species at any dose level.

Experimental animals other than the mouse have been studied relatively little for radiogenic leukemia. Spontaneous and induced myeloid and lymphocytic leukemias have been reported in rats (13, 14) and lymphatic leukemia in irradiated guinea pigs (15), but the relation of these growths to radiobiological and other factors remains largely unexplored.

Dose-Response Relationship

The precise relation between leukemia incidence and dose is poorly defined as yet, especially for low-level irradiation. Also poorly known

TABLE 1.—Leukemia incidence in RF/Up mice irradiated *in utero*

X-ray dose (r)	Age at exposure (days)‡	Number of mice†	Leukemia (%)*	
			Myeloid	Thymic lymphoma
0	Control§	1, 211	1. 0	4. 0
50	9. 5	243	1. 5	3. 5
50	12. 5	235	2. 0	3. 5
50	17. 5	238	1. 0	4. 5
150	9. 5	234	1. 0	4. 5
150	12. 5	237	1. 5	1. 5
150	17. 5	238	1. 0	2. 5
300	17-20	36	0	0

*Preliminary analysis—youngest mice 18 months old.

†Number surviving over 10 weeks of age (equal number of males and females).

‡Age (days) post conception.

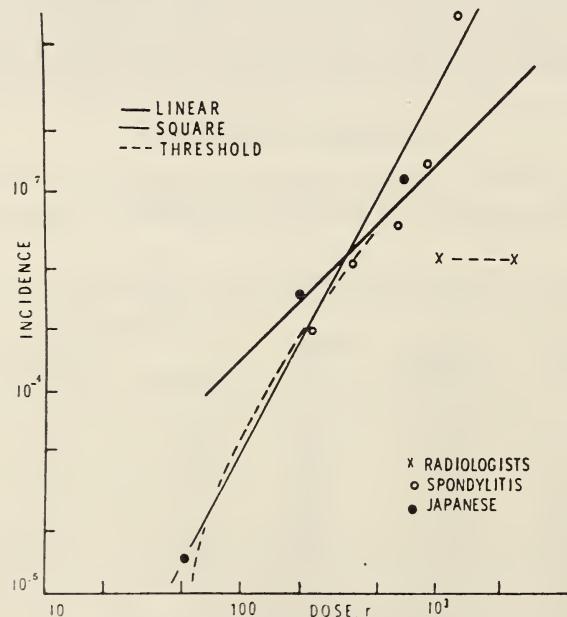
§Controls include mice sham-irradiated at 9.5, 12.5, and 17.5 days.

||Data from Upton *et al.* (73).

are the effects of partial-body, as opposed to whole-body irradiation and the influence of other radiologic variables; *i.e.*, linear energy transfer (LET), dose rate, and dose fractionation (16).

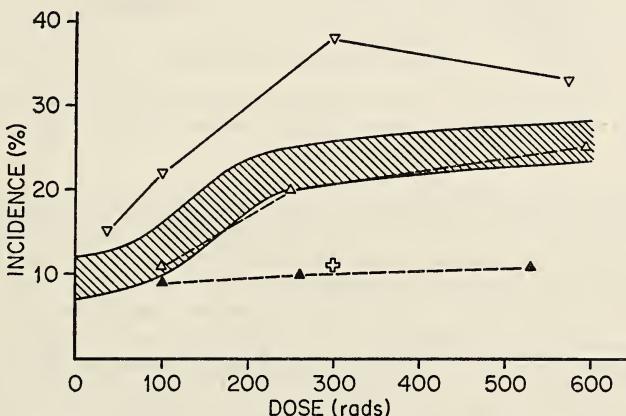
The dose-incidence relation in man has been analyzed by several investigators, with varying interpretations. In short, the available data do not provide an unambiguous picture of the dose-response relationship (text-fig. 7). They suggest, however, that long-term irradiation over many years (as in the case of U.S. radiologists) is less leukemogenic than short-term or instantaneous irradiation (as in the irradiated spondylitics and A-bomb survivors) and that the dose-incidence relation is probably exponential rather than linear, although the latter possibility cannot be excluded (17-19), especially in the case of exposure *in utero* (20). The evidence from spondylitics and from children irradiated over the mediastinum in infancy (21, 22) suggests, furthermore, that it is not necessary to irradiate the entire body to induce leukemia in man, although the influence of volume-dose relationships is not clear.

Dose-incidence studies in the mouse reveal a complex interaction among radiologic and host factors, as well as variation with the hematologic type of leukemia in question. The most widely studied form of murine leukemia is thymic lymphoma. The dose-incidence curve for this neoplasm is characteristically sigmoid, and its slope varies with the age, sex, and strain of the population exposed (19). A given dose of whole-body X rays or γ rays is generally more effective when applied in a single brief exposure than when applied at a low dose rate or in many



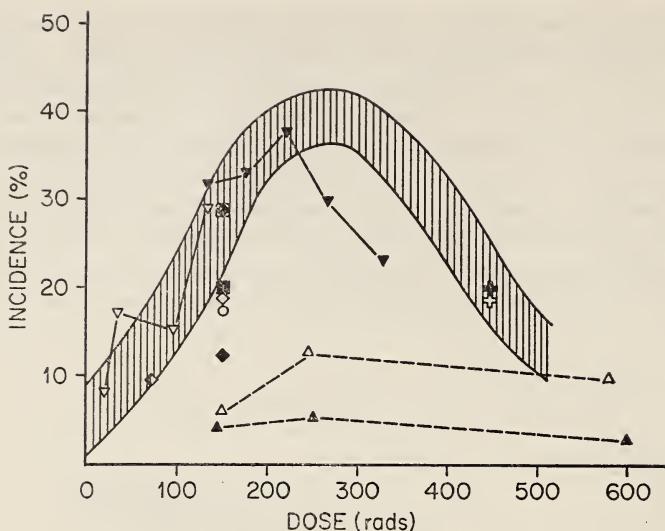
TEXT-FIGURE 7.—Relationship between leukemia incidence and radiation dose in irradiated human populations. The curves illustrate three alternative functions fitted to the same data. From (82).

successive exposures (7), but properly fractionated treatments may be more oncogenic to the thymus than a single exposure (23). These relationships are illustrated in text-figure 8, which also shows that there is a marked difference in the intensity-dependence of neutrons and γ rays, the leukemogenic effectiveness of fast neutrons remaining relatively high at low dose rates. Also illustrated in text-figure 8 is the lack of leukemia induction in mice protected by marrow shielding, which is in agreement with earlier observations indicating that the presence of intact hematopoietic cells protects against radiogenic lymphomas and that the thymus itself need not be irradiated if all other hematopoietic tissue is exposed (24, 25).



TEXT-FIGURE 8.—Incidence of thymic lymphomas in RF/Up female mice exposed to various types of whole-body and partial-body radiation, beginning at 10 weeks of age. \triangle $\text{Co}^{60}-\gamma$ rays, whole-body, 23 hours daily (0.01–0.012 rad/min); \blacktriangle $\text{Co}^{60}-\gamma$ rays, whole-body, 23 hours daily (0.00036–0.0036 rad/min); white + $\text{Co}^{60}-\gamma$ rays, one third of body exposed, single exposure (7 rads/min); ∇ Po-Be fast neutrons (~ 5.0 Mev avg energy), whole-body, 23 hours daily (0.00058–0.0012 rad/min). Shaded area indicates results of a single exposure to whole-body $\text{Co}^{60}-\gamma$ rays (7–50 rads/min), with 95 percent confidence limits.

The induction of myeloid leukemia has been investigated less intensively than the induction of lymphomas, since it is relatively less common in most strains of mice. Available data (text-fig. 9) on the induction of myeloid leukemia in RF/Up mice suggests the following: 1) The dose-incidence curve is sigmoid below 250 rads for acute X or γ irradiation; 2) γ rays are less effective at low than at high dose rates, but 2 to 3 successive high-intensity exposures are markedly additive; 3) there is little, if any, dose-rate dependence for fast neutrons; 4) exposure of one half or two thirds of the body is leukemogenic, although fewer leukemias are induced than when the remainder of the body is exposed concomitantly to the same incident dose; and 5) above 250 rads, the incidence decreases with increasing dose. In limited experiments to date, radioactive strontium deposited internally in doses that increase the incidence of lymphomas has failed to alter the incidence of myeloid leukemia in RF mice (26).



TEXT-FIGURE 9.—Incidence of myeloid leukemia in RF/Up males exposed to various types of whole-body and partial-body radiation, beginning at 8 to 10 weeks of age. Shaded area indicates results of a single exposure to whole-body X or γ rays (7-80 rads/min), with 95 percent confidence limits. \circ Two whole-body X-ray exposures of 75 r (80 r/min), interval of 2 days between exposures; \bullet 2 whole-body X-ray exposures of 75 r (80 r/min), interval of 6 days between exposures; \blacksquare 2 whole-body X-ray exposures of 75 r (80 r/min), interval of 7 days between exposures; \square 2 whole-body X-ray exposures of 75 r (80 r/min), interval of 28 days between exposures; $\text{white} + 3$ whole-body X-ray exposures of 150 r (80 r/min), interval of 5 days between exposures; \blacktriangle 3 whole-body X-ray exposures of 150 r (80 r/min), interval of 5 days between exposures; \triangle $\text{Co}^{60}\gamma$ rays, whole-body, 23 hours daily (0.007-0.07 r/min); \blacktriangle $\text{Co}^{60}\gamma$ rays, whole-body, 23 hours daily (0.00072-0.0036 r/min); \blacktriangledown cyclotron fast neutrons (~ 1.0 Mev avg energy), whole-body, single exposure (50-120 rads/min); ∇ Po-Be fast neutrons (~ 5.0 Mev avg energy), whole-body, 23 hours daily (0.00058-0.0056 rad/min); \lozenge X rays (80 r/min), 75 r to upper half of body followed 7 days later by 75 r to lower half of body; \diamond X rays (80 r/min), 75 r to upper half of body followed 7 days later by 75 r to whole-body; \blacklozenge X rays (80 r/min), 150 r to upper two thirds of body.

If the dose-response relationships for leukemia induction in man and the mouse are compared, the data for murine myeloid leukemia appear to approximate more closely those for human leukemia than do the data for thymic lymphomas. The decline in the frequency of myeloid leukemia with increasing dose above 300 rem is, however, peculiar to the mouse. Although unexplained, it may reflect decimation of the population-at-risk by other causes of death at high radiation dose levels, since the incidence of myeloid leukemia becomes high enough at 300 r (30-40%) to make the curve sensitive to such selection pressures (8). The estimated rate-doubling dose for this disease in the mouse appears similar to that in man (*i.e.*, 50-100 rem acute whole-body radiation).

All considered, the dose-response data suggest that leukemia induction requires a multiplicity of effects rather than a single, one-hit type of

radiobiological change. This interpretation is not inconsistent, however, with the somatic mutation hypothesis of oncogenesis in view of recent observations concerning the influence of time-intensity factors on mutagenesis in germ cells (27-30). Hence, further quantitative studies of leukemogenesis in relation to mutagenesis and related effects on chromosomes are called for.

Role of Chromosome Changes

The consistent presence of the Philadelphia chromosome in patients with chronic myeloid leukemia (31) has given impetus to the search for chromosomal aberrations in other types of neoplasia. As yet, however, no other single karyotypic abnormality has been found in constant association with a specific neoplasm. Furthermore, many clinical and experimental growths appear karyotypically normal, although it is possible that they have minor chromosomal changes not detectable by existing morphologic techniques. The significance of the Philadelphia chromosome is, therefore, of great interest, especially since it may be present in blood cells other than leukemic leukocytes, which suggests that it denotes a change in a common blood cell progenitor (31).

Attempts to analyze the frequency of spontaneous and radiogenic leukemia in the light of existing information on the rate of chromosome breakage suggest that either the Philadelphia chromosome is not a simple deletion or is not *per se* responsible for the development of chronic myeloid leukemia; *i.e.*, the calculated rates of spontaneous and radiogenic deletions which might be expected to give rise to a Philadelphia chromosome, and, in turn, to chronic myeloid leukemia, exceed by several orders of magnitude the observed natural and radiation-induced incidences of the disease (33). This is consistent with the interpretation that the pathogenesis of leukemia involves more than a single change in the cell (17) and that the Philadelphia chromosome is, therefore, only part of a more complex abnormality in the affected cell or else a consequence rather than a cause of the leukemic transformation.

Preliminary observations on a serially transmitted radiogenic myeloid leukemia in RF/Up mice have disclosed in this disease a consistent karyotypic defect in marrow cells, consisting of an increase in the modal number of chromosomes to 41, associated with a distinctive chromosomal marker (N. H. Wald and A. C. Upton, unpublished data). As with the Philadelphia chromosome, this abnormality has been noted in all affected mice studied to date, whether they received the disease through inoculation of cells or cell-free extracts. However, the etiologic significance of the lesion remains to be determined, especially since primary cases of radiogenic leukemia have yet to be examined cytologically. The character of the marker chromosome suggests that a small autosome may have been added to one of the large chromosomes by translocation, giving rise to trisomy for this small autosome. If this interpretation proves correct, the karyotypic abnormality may conceivably be analogous to that in Down's

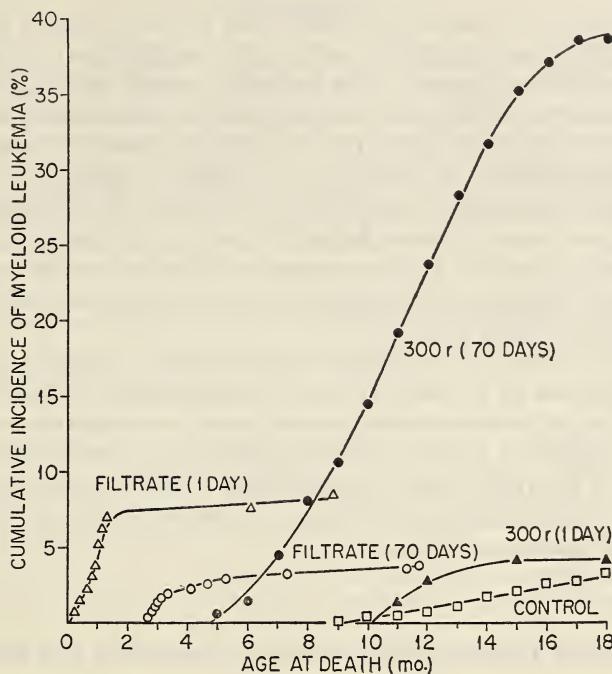
disease, *i.e.*, trisomy for a minute autosome, presumably chromosome 21, which greatly predisposes to leukemia (33).

Role of Viruses

Discovery of viral agents in the etiology of fowl leukemias dates from the beginning of the tumor virus era (34). The more recent demonstration by Gross of the susceptibility of the newborn mouse to leukemia viruses has led to implication of viral agents in the pathogenesis of spontaneous and radiogenic leukemias in mice (32, 34, 35). Certain of these viruses, moreover, are leukemogenic for newborn rats (36, 37). Data on other species are fragmentary and inconclusive. Although virus-like particles have been noted infrequently in the tissues of leukemic patients (38) and cell-free extracts of such tissues have been reported to transmit leukemia to experimental animals (34), the significance of these findings for the pathogenesis of human leukemia is uncertain.

The mode of action of known tumor viruses in oncogenesis and the role of radiation and other carcinogens in "activating" such agents are obscure. The problem is further complicated by the observation that viruses heretofore considered nononcogenic may exert carcinogenic or cocarcinogenic effects in experimental animals under certain circumstances (39-42). Speculations on the respective roles of viruses, radiation, and other factors in leukemogenesis have been published elsewhere (32, 43, 44). To these must now be added the possibility that viruses may exert oncogenic effects through the induction of chromosomal aberrations, a mechanism postulated to account for the neoplastic transformation of hamster cells by polyoma virus *in vitro* (45) and one which may conceivably operate *in vivo* in view of the chromosome-breaking action of measles virus (46) and the induction of the specific chromosomal abnormality by cell-free leukemic extracts mentioned in the foregoing.

Whatever respective roles radiation and virus may play in leukemogenesis, the relationship between latency and final incidence differs markedly in irradiated, as compared with filtrate-injected, mice (text-fig. 10). This difference suggests that the effects of radiation involve more than the immediate "activation" of a latent leukemia virus. A similar conclusion is suggested by the observation of Kaplan (24) that the leukemogenic effects of four successive weekly exposures to X rays may be largely nullified by implantation of intact isologous bone marrow cells after the last exposure. Likewise, the work of Berenblum and Trainin (47) suggests that the leukemogenic action of radiation includes "promoting" as well as "initiating" effects. Although the former may be postulated to involve the activation or liberation of a latent transmissible factor which can initiate leukemogenesis but which alone is not fully oncogenic, the "promoting" effects which complete the leukemogenic process (47) are yet to be characterized. They may conceivably entail: 1) alteration of host susceptibility through interference with thymus and marrow cell renewal and maturation, as postulated by Kaplan (24); 2) depression of



TEXT-FIGURE 10.—Cumulative incidence of myeloid leukemia in RF/Up male mice, as influenced by irradiation or inoculation of leukemic filtrates.

□ Untreated controls; ● 300 r whole-body X rays at 70 days of age; ▲ 300 r whole-body X rays at 1 day of age; ○ inoculation of cell-free filtrate of myeloid leukemic spleen at 70 days of age; △ inoculation of cell-free filtrate of myeloid leukemic spleen at 1 day of age.

antiviral immunity; 3) production of mutant cells with heightened susceptibility to virus; or 4) combinations of these and other mechanisms. That irradiation may increase the responsiveness of mice to oncogenic viruses has been demonstrated with several different viruses and in various host strains (48-50).

OTHER NEOPLASMS

In addition to certain types of leukemia, other neoplasms have been noted to occur with increased frequency in irradiated human and animal populations. These include: 1) cutaneous carcinomas and sarcomas (2, 51); 2) osteosarcomas (52-54); 3) pulmonary and bronchial carcinomas (55, 56); 4) thyroid adenomas and carcinomas (57-59); 5) sarcomas and carcinomas of the liver (60-63); and 6) miscellaneous tumors of other sites (1, 3).

It is beyond the scope of this survey to discuss these neoplasms in detail. In general, however, the evidence suggests that carcinogenic effects of radiation in man and in laboratory animals are qualitatively similar,

although there are quantitative differences between species and strains in susceptibility to oncogenesis and in the predilection of the induced tumors for different organs. For example, certain growths commonly induced in mice of most strains, *i.e.*, thymic lymphoma and granulosa cell tumor of the ovary, are rarely, if ever, induced in man. The relationship between susceptibility to induction of a given neoplasm by irradiation and the risk of its natural occurrence in any given species remains to be established, as does the basis for differences in the latency of different types of growths within a given species or between species (*e.g.*, see 19, 64, 65). Speculations on this subject have been numerous (66) but definitive experiments have been few.

Possibly relevant to the question of latency is the tendency for chromosomal aberrations to increase with age in animals (67, 68) and in human beings (69) and the observation that radiogenic chromosomal aberrations persist for months or years in various types of cells *in vivo* (70, 71). Further study of the relationship of such effects to age, life span, and cellular turnover in various species may help to elucidate aspects of comparative oncogenesis hitherto obscure.

Comparative Effects of Radiomimetic Chemicals and Radiation

Many of the oncogenic effects of ionizing radiation are reproduced by radiomimetic alkylating agents of various types (table 2), but there are quantitative, and possibly qualitative, differences in the effects of the various agents on different organs and cells. Furthermore, the effects of radiation in combination with chemical carcinogens do not always appear additive (16, 72), which suggests that the agents may differ in mode of action.

TABLE 2.—Late somatic effects of radiomimetic chemicals

Effect	Species	Reference
Shortening of lifespan	Mouse	Alexander and Connell (74) Upton <i>et al.</i> (75) Nemeth (76)
	Rat	Dunjic (77)
	Mouse	Upton <i>et al.</i> (78) Nemeth (76)
Carcinogenesis	Mouse	Conklin <i>et al.</i> (79) Nemeth (76)

Since systematic dose-response studies of the interaction of radiation and chemicals have yet to be carried out, and because the uptake and metabolism of carcinogenic chemicals at the subcellular level are not yet well characterized, a searching analysis of comparative mechanisms of carcinogenesis by radiation and chemicals must be deferred. It may be hoped, however, that work on this question will be intensified in the near future because of its theoretical and practical importance.

RESUMEN

Las radiaciones parecen ser carcinogénicas para todas las especies de mamíferos estudiadas hasta el presente. Las especies varían, sin embargo, ampliamente en la posibilidad de inducir neoplasias de algún órgano o tipo celular, y dentro de una misma especie hay variaciones en susceptibilidad de acuerdo a la cepa, al sexo, a la edad de irradiación, a la condición fisiológica y a otros factores. La relación entre dosis de radiación y probabilidad de neoplasia es compleja y sugiere, en general, que la transformación neoplásica involucra más bien un proceso de múltiples etapas en lugar de una sola alteración del tipo de golpe. La radiación de baja energía linear transferida (LET) es generalmente más efectiva cuando se administra a una alta intensidad que cuando se administra a una baja intensidad; la radiación de alta-LET es, en general, relativamente menos dependiente de la dosificación y más efectiva que la radiación de baja-LET. Muchos de los efectos carcinogénicos de la radiación son reproducidas por substancias químicas radiomiméticas, en especial para aquellas conocidas por ser mutagénicas y con potencia para romper los cromosomas; sin embargo, los efectos combinados de radiación y agentes químicos no son necesariamente aditivos, lo cual sugiere que los agentes pueden diferir en su modo de acción. La evolución de la neoplasia además parece requerir en muchos casos de la interacción de las células formadoras de cancer con los factores extrínsecos promotores de cancer en su ambiente, y en algunos casos las mismas células formadoras de cancer no necesitan ser irradiadas. Por lo tanto, el significado de los efectos mutagénicos en la carcinogénesis no está claro. En la patogenia de algunas neoplasias inducidas por radiación parecen estar comprometidos los virus oncocáncer, cuyo origen y papel están aun por definirse.

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DISCUSSION

Kaplan: This comment may not apply to the myeloid leukemias, but certainly to the thymic lymphomas, in which an indirect mechanism has been established; in that instance, if a mutation is involved at all, it is certainly not produced by radiation. Now, whether the virus produces mutation or not is a separate question. I think, however, it is no longer warranted for us to continue to look seriously at the problem of mutation by radiation as a mechanism of carcinogenesis in this particular disease.

Upton: I could not agree more completely, Dr. Kaplan; sorry I did not make that point clear in my presentation.

As concerns the time relation between radiation and injection of filtrate in the experiments with brain filtrate, we injected the filtrate 24 hours before irradiation. The experiments with spleen filtrate were, in one case only, carried out by irradiation 24 hours before injection of filtrate. We are now irradiating filtrate-inoculated newborn animals at 10 weeks of age. These are animals that failed to get leukemia as a result of the injection of filtrate on the very 1st day of life. As yet, the results are fragmentary, and we have only a few animals in the experiment. We wonder whether injecting them with big doses of virus on the 1st day of life may render them more or less susceptible to later irradiation leukemogenesis.

Kaplan: I am glad to have this information because it fits in with some notions that I shall present. In trying similar procedures with strain C57BL mice, we have given virus filtrates several days ahead of or several days after irradiation. In both instances, we have repeatedly and convincingly demonstrated synergism. Synergism is greater if the virus is given several days before irradiation. I will reserve our interpretation until later. I would encourage you to repeat the experiment with better or more advantageous spacing of the two treatments.

Rogers: Several years ago we did some work relating to why animals get tumors when they become old rather than when they are young, at which time one may more readily induce tumors with carcinogens, such as urethane. I think this has subsequently also been tested with urethane by Dr. Tannenbaum. We put young mouse tissues in old and young mice. Lung tissue was put in leg muscles and, after a period of months, examined to find which set of animals had more tumors in their implants. We found no difference whether the tissue resided in an old or a young mouse. There-

fore, the spontaneous occurrence is a function of the age of tissue rather than the age of the host. On this basis, and because of work with X rays with which, in contrast to leukemia effects, you get no increase in lung tumors except with chronic radiation over a long period, it seemed possible that precise time of effectiveness of X-ray treatment during the cell mitotic cycle is so brief that the number of susceptible cells is too low to induce a significant number of tumors with a single exposure of X rays. Therefore, we put lung tissue in the leg muscle of mice and injected the implants with radioactive gold, giving the animals at the site of the implant 1000 r over a period of about a week. Even under these circumstances we got no increase in pulmonary adenomas in the implants. It thus appeared quite possible that what X rays were doing in chronic radiation was simply to kill off certain cell families; the others had to replace them and, thereby over a long period greatly increased the number of cell divisions of any given cell family and that this was the critical factor in pulmonary tumors occurring spontaneously or following chronic exposure to X irradiation.

Another point concerns the occurrence of the Philadelphia chromosome in certain leukemias. One wonders whether nontumor viruses (for example, the measles virus) might transduce information out of the center of a chromosome leaving a gap, causing chromosome breakage and loss of a part, perhaps a growth repressor—this loss being the cause of the leukemia and the virus only fortuitously related.

Mole: Dr. Upton, are you really satisfied that there is any good evidence that the human fetus is appreciably more sensitive than the human adult? The records on children irradiated *in utero* suggest fairly strongly that the whole of the leukemia that is going to occur will have occurred by the tenth birthday. In Japan in bomb survivors at Hiroshima and Nagasaki and in Britain where older people with ankylosing spondylitis have been irradiated, leukemia is still occurring 10 and 15 years later so that we do not know yet how much leukemia is going to be produced in the adult. Yet another point is that the fetal exposures, dealt with in Dr. Stewart's and Dr. MacMahon's surveys, were those of 15 years ago when the doses given individuals must certainly have been greater than those given today, because in the intervening time there have been improvements in the manufacture of X-ray film. X-ray films are better now than they were 15 years ago. There is also, of course, in the last 10 years an appreciation that radiation might do harm, and I think therefore a tightening up of technique in antenatal radiography. Considering these points, I think that there is no real evidence that the human fetus is more sensitive than a human adult.

Upton: I do not feel really competent to discuss this particular point thoroughly; it is not an aspect of leukemogenesis that I have investigated personally in detail. It would seem to me that, from the data available, one would have to see a great many more leukemias in adults to suggest that there is no age difference in susceptibility. On the other hand, the leukemias in children are chiefly of a different hematologic type. It may be a type difference. I would like to hear comments from Dr. Kaplan and Dr. Court Brown who have published studies on this question.

Kaplan: I always enjoy quarreling with my friend, Dr. Mole, but he seldom gives me an opportunity to do so successfully, as I think he now has. It seems to me his statement is a logical *non sequitur*. He has attacked Dr. Upton for the statement that there is an enhanced sensitivity or susceptibility of the fetus to radiation because the data are based on 15-year-old X rays! The fact is, that based on those data, there was an enhanced susceptibility. It is small, but it seems reasonably well demonstrated by a number of studies, all of which taken together indicate something like a 40- or 50-percent increase in the frequency of childhood leukemia. The second thing Dr. Mole said is that this is not necessarily correct because all of the adult leukemias have not been harvested, but this implies that all leukemias at all ages are the same, which point he himself has gone to some pains to prove is not correct. You cannot lump the adult leukemias with childhood leukemias.

Finally, Dr. Mole said that the danger may be mitigated by the fact that today we have faster X-ray films and we are more aware of the hazard. This may reduce the amount of leukemia we produce in fetuses, but it does not change the fact that the fetus is in-

herently more susceptible. So I would like to repeat that Dr. Upton's statement is correct and Dr. Mole's is a logical *non sequitur*.

Mole: I feel there really are some misunderstandings here because I accepted the increase of 40 or 50 percent in children as a consequence of antenatal radiography 15 or more years ago. I was merely questioning the statement that the irradiated human fetus is more likely to get leukemia than human adults, but I do not think anything you said bears on that subject.

Rogers: In relation to the virus and the latent period, I would like to raise the question of whether or not we might use the latent period in virus infections—such as is associated with thymic lymphoma or myelogenous leukemia—as a means of indicating whether or not the action of virus is direct or indirect. A long latent period occurs with polyoma virus and during this period (Vogt and Dulbecco) there is much chromosome breakage and finally neoplastic transformation. As shown by others, such as Defendi, the virus-specific antigens occurring in polyoma virus-infected cells appear very early after infection of the virus and long before the neoplastic transformation. These characteristics of the polyoma virus are in great contrast to Shope papilloma virus, in which the tumor occurs at once after infection of the cell and antigens, which we and others have been able to find in it, also occur as early as we can pick up the tumor. It seems therefore that with some of these viruses we might use the latent period as an indication that the genetic information on the virus does or does not pertain directly to the neoplastic transformation.

Hueper: Dr. Upton, do you ascribe to other cancers you have observed in your radiation studies besides leukemia a viral causation? We have quite a bit of information on chemical carcinogenesis in which one can get multipotent tumors with one chemical carcinogen. If there is an analogy between chemical carcinogenesis and radiocarcinogenesis, I wonder whether one can conclude that lung cancer and ovarian cancer are possibly due to a viral origin?

Upton: You have raised an interesting and important question, Dr. Hueper. I have to confess that we have not systematically looked for viruses in tumors other than leukemia. Certainly we have evidence that the polyoma virus is more effective in irradiated animals than in nonirradiated animals and induces a wide variety of types of neoplasms. Hence we have a theoretical basis for suspecting that tumors of many organs can be produced through the action of viruses. At the moment we badly lack potent assay techniques. The work I mentioned, which looks pitifully meager, summarizes in a few minutes what has taken literally 10 years to compile. I think that any attempts to look for viruses in radiogenic tumors are going to be very difficult and will require sophisticated and intensive approaches. What we will say 10 years hence, I do not know. I would have guessed 10 years ago that radiation leukemia is not a virus disease. I remember very well the discussions of Gross's original publications. An immense amount of skepticism prevailed at that time. I am not a virologist. I am not trying to imply that all cancer is virogenic, but the possibility exists. Personally, I am inclined to think that cancer is not one disease, but many, and results from many different kinds of mechanisms.

Hoecker: What is significant to an outsider is that all that radiation does is to increase what normally seems to go on, and I would like to ask Dr. Upton what his views are on this very striking relation.

Upton: The tendency for cancer to occur with increasing age has prompted many speculations as to the sequence of events that must occur in an individual to make for the development of a tumor. I must confess that, at the moment, I am inclined to look for changes in the genetic or epigenetic information in the cell. It certainly is well known that mutations accumulate with time and under the influence of radiation. Radiation can activate viruses or render cells, possibly by mutation, susceptible to viruses. To that extent, radiation does compress, or telescope, the process with respect to time. This problem of latency is a big problem, however; as yet I do not know of any completely satisfactory explanation for latency.

Mole: I intend again to take up this point as to whether it is the least bit surprising that radiation produces just those things which occur naturally. I would have thought there must be only a limited number of ways in which cells can respond, and that the nature of the agent starting off the response is irrelevant to the end product. Thus, the only kind of tumor you can get in the lung for example must be a lung tumor, not a tumor of ovarian cells or brain cells. One would not expect any qualitatively different response from methylcholanthrene, radiation, or anything else.

Leblond: I am surprised that there has not been more of an attempt made to reduce the normal radiation level. Dr. Figge, in the United States, and Dr. Frank, in Canada, compared cancer incidence in mice kept in deep mines and in others kept under usual laboratory conditions. They found little difference, but they eventually realized that there was as much radiation in these mines as at the surface. I wonder why there has not been an attempt to work in low-background rooms that need not be in mines, but that would be designed to reduce the background to less than 25 percent of the natural radiation. Perhaps then cancer incidence could be lessened.

Upton: This approach is always attractive at first glance, but it turns out that relatively enormous amounts of radiation are required for detectable oncogenic effects in animals, as compared with natural background levels. We and others have been unable to detect tumorigenic effects with low-level irradiation in fairly sizable populations. In our experience, for example, with 0.5 r per day of γ -rays given over 23 hours a day, we are unable to see any significant life shortening and oncogenesis in mice, yet this is at least 1,000 times more radiation than they receive naturally. I think it is quite unlikely therefore, short of astronomical numbers of animals, that one can get at the problem by trying to reduce the background radiation level. Some years ago, Failla (Failla and McClement, Amer J Roentgen 78: 946, 1957) calculated that, based on the amount of life shortening resulting from increments of radiation on the natural background, mice age naturally at a rate corresponding to about 12 rem per day, which is far more radiation than they would get from natural background. I think that one would have to conclude that radiation is only one of many factors in the environment, as well as intrinsic factors, which cause aging and neoplasia.

Parameters of Radiation Dosage That Influence Production of Osteogenic Sarcomas in Mice^{1, 2}

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SUMMARY

A wide background of information has made it possible to predict, with some degree of confidence, the carcinogenic response of mice to bone-seeking radionuclides provided the test animal is a 70-day-old CF1 female and the test material is given in a single, intravenous injection. This paper describes five current experiments in various stages of completion in which some of these conditions were altered. A single injection of Sr⁹⁰ and of Ca⁴⁵ is compared with 5 and 20 fractional injections, as well as with continuous ingestion. Continuous exposure to a radioactive diet from the time of conception is compared in

female and male mice, and exposure of females from conception is compared with exposure starting at about 150 days of age. Fractional injection and continuous ingestion change the irradiation pattern that follows a single injection by providing uniform distribution of the dose in space and in time. The opposite approach is to deliver a dose that is limited in space and in time. Time limitation is being studied by use of Y⁹⁰, which has a very short half-life, and by exposure of the hind legs alone to carcinogenic doses of X rays.—*Nat Cancer Inst Monogr* 14: 243-270, 1964.

THE CARCINOGENICITY of radionuclides that may gain entrance into the body is related to certain of their physical and chemical properties. In producing bone cancer by radioactive materials that localize in the skeleton, alpha-emitters are more effective than beta-emitters, strong beta rays are more effective than weak beta rays, and isotopes with half-lives measured in weeks, months, or years are more effective than isotopes with very short half-lives. Knowledge of these relationships and others associated with the daughter products of radioactive decay has made it possible to predict the carcinogenicity of untested bone-seeking isotopes under a set of standard conditions; these include the administration of the radionuclide in a single intravenous injection to 70-day-old CF1 female mice.

With this information as a foundation, we are now studying the production of osteogenic sarcomas in mice when the standard conditions are

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changed. In the experiments described here, we gave particular attention to the association between several factors of radiation dosage—intensity, duration, and magnitude of exposure—and the carcinogenic response. The five major experiments included in this report are at several stages of completion: One group is completed, two require only histopathologic study, but in the remaining two a large proportion of the animals is still alive. The reliability of comparisons and the soundness of interpretations possible at this time depend, of course, upon the degree of completion of the studies. However, except for the last two, the trends of the results are already firmly established, and adding or subtracting a few osteogenic sarcomas as the data become complete will not change the general picture substantially. Therefore, we have taken advantage of the excellent opportunity provided by this Symposium to review and compare the available results of these investigations.

The experiments will be discussed under (a) fractional exposure, (b) continuous exposure, and (c) time-limited irradiation. In *fractional exposure* the total amount of Sr⁹⁰ or Ca⁴⁵ was given in 1, 5, or 20 injections. This procedure changed the temporal and spatial pattern of absorbed dose without changing the total dose received by the mouse. In *continuous exposure* the mice were maintained as long as they lived on food with a constant Sr⁹⁰ to calcium ratio. This procedure provided a gradual accumulation of Sr⁹⁰ in the body and assured a more uniform spatial distribution of the dose. In *time-limited irradiation* the carcinogenic agent used was either Y⁹⁰, which has a physical half-life of 64 hours, or X rays. These sources permit manipulations of dose rate and duration that are not possible with longer-lived radionuclides.

EXPERIMENTAL

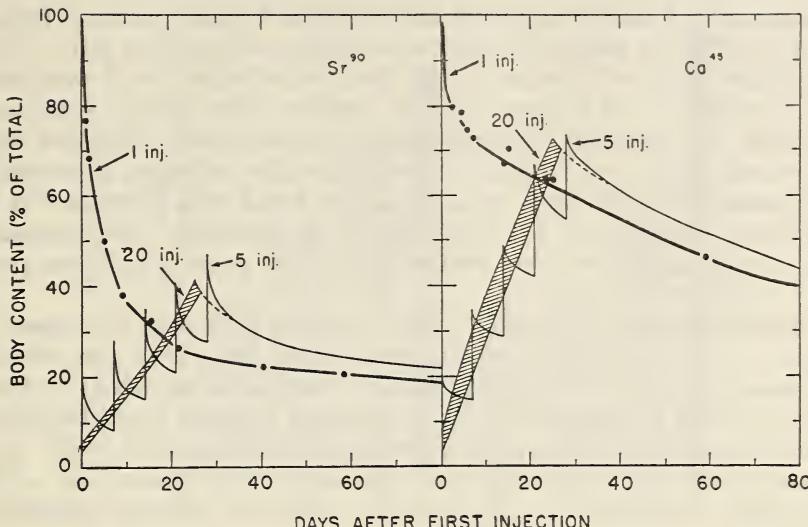
Fractional Exposure

In the fractional exposure experiments Sr⁹⁰ and Ca⁴⁵ were injected intravenously, the total amount being given either in a single injection, in 5 partial injections once a week for 5 weeks, or in 20 partial injections 5 times a week for 4 weeks. Three levels of each nuclide were used: 1.0, 0.5, and 0.25 μ c of Sr⁹⁰ per g (in equilibrium with Y⁹⁰), and 6.0, 4.0, and 2.0 μ c of Ca⁴⁵ per g. There were 510 mice given Sr⁹⁰, 300 given Ca⁴⁵, and 135 controls that provided information on osteogenic sarcomas and an additional number that were used for autoradiography, whole-body counting, and radioanalysis.

Results

Body burden

The amount of Sr⁹⁰ and Ca⁴⁵ in the body under the 3 regimens of injection is shown in text-figure 1. Initially there was a substantial difference in body burden depending upon the number of injections, but the difference



TEXT-FIGURE 1.—Body burden of Sr^{90} and Ca^{45} as a function of time after intravenous administration. Total amount was given either in 1 injection, in 5 injections at the rate of 1 a week, or in 20 injections at the rate of 5 a week. *Shaded area* includes the minimum and maximum when 20 injections were given.

had essentially disappeared by 20 days. After the last injection, which was on day 28 for 5 fractions and day 25 for 20 fractions, the animals that had received multiple injections contained more radioisotope than singly injected animals. This difference decreased with increasing time.

The retention of Ca^{45} was much higher than the retention of Sr^{90} , and, were it not for the short half-life of Ca^{45} (165 days), the dissimilarity between the two curves would have been even larger. Because of this unequal retention, comparisons of body burdens of Sr^{90} and Ca^{45} when given in 1, 5, or 20 injections show somewhat different relationships. For example, the maximum Sr^{90} burden after 5 injections was 48 percent of the maximum after a single injection, as compared to 74 percent for Ca^{45} .

Autoradiography

The three injection schedules produced different patterns of deposition of the radionuclides. Autoradiographs of femurs of mice that had received Sr^{90} appear in figure 1. These pictures were prepared from thin bone sections, 50 to 80 μ thick, so that autoradiographic localization would be distinct.

Autoradiographs showed: 1) The deposition of Sr^{90} was very heavy in the distal epiphyseal plate and adjacent metaphysis during the 1st week after injection because the bone was still growing. 2) Sixty-three days after the injection of 0.5 μc per g the metaphyseal Sr^{90} had disappeared. 3) Even 100 days after the injection of 1.0 μc per g a heavy deposit remained in the

metaphysis. Vaughan and Owen have reported a similar failure of resorption of Sr^{90} in the rabbit tibia after the injection of $0.6 \mu\text{c}$ per g (1). This particular femur is 2 percent longer than those taken 5 to 8 days after injection, but it is 2.4 percent shorter than the other 100-day specimens. Possibly the retention of metaphyseal strontium was associated with decreased growth. 4) A band of autoradiographic darkening appeared in the metaphysis at 63 days when $0.5 \mu\text{c}$ per g had been given in 5 or 20 fractional injections in a 4-week period. 5) Generally, the autoradiographic appearance was very similar whether $0.5 \mu\text{c}$ per g had been given in 5 or 20 fractions.

Autoradiographs of femurs of mice that had received Ca^{45} appear in figure 2. Specimens from mice that had received $6.0 \mu\text{c}$ per g were selected for autoradiography for practical reasons based upon the half-life of Ca^{45} . However, had we known that the carcinogenic response would be greater after the injection of $4.0 \mu\text{c}$ per g, we would have used that level. It is possible that the smaller response at the higher level is associated with a metabolic disturbance of the bone that could be reflected autoradiographically. However, in a previous study (2) the proportion of injected Ca^{45} measured autoradiographically in mouse femurs was independent of the amount given over a range of 0.25 to $6.0 \mu\text{c}$ per g.

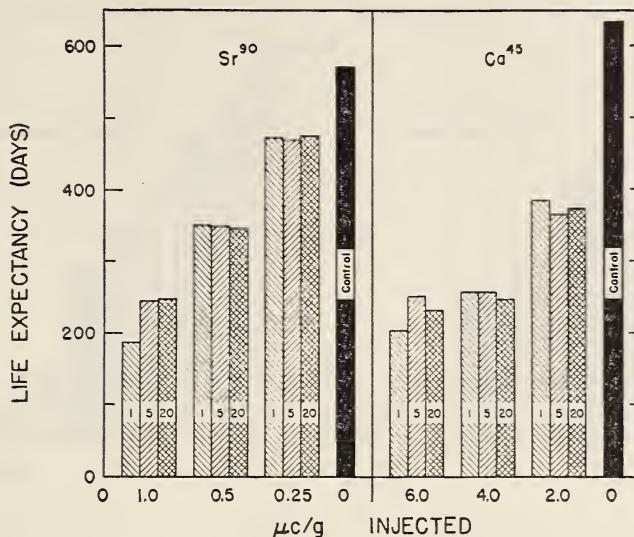
The following features of figure 2 are of particular interest: 1) The localization of Ca^{45} 4 hours after injection was similar to the early localization of Sr^{90} . 2) Twenty-eight days after injection the distal metaphysis did not appear on the autoradiograph, and the distal epiphyseal plate was much less intense than the cortex. 3) The autoradiographic appearance of the femur 58 days after injection was the same as it had been at 28 days. 4) Four hours after the last of 5 or 20 fractional injections the darkening of the epiphyseal plate was very dense. 5) At 58 days the darkening of the epiphyseal plate was still very dense if 5 fractional injections had been given. However, autoradiographic darkening was much less intense if 20 fractional injections or a single injection had been given.

Lifespan

Life expectancy of each group of isotope-treated animals was less than the life expectancy of the controls (text-fig. 2). Except for $1.0 \mu\text{c}$ of Sr^{90} per g, it made little difference into how many fractions the total amount had been divided. Lifespan decreased as the amount of Sr^{90} increased, but $4.0 \mu\text{c}$ of Ca^{45} per g had as great an effect as $6.0 \mu\text{c}$ per g.

Osteogenic sarcomas

The incidence of osteogenic sarcomas and the expectancy of death with these neoplasms are presented in text-figure 3. The 600 Sr^{90} mice had 1,119 malignant bone tumors, and the 345 Ca^{45} mice had 525. Tumor incidence, calculated by dividing the total number of tumors by the total number of mice, is the customary value, but tumor expectancy provides a more reliable index of carcinogenicity because it is not as seriously influenced by incidental deaths that may reduce a population quite early.

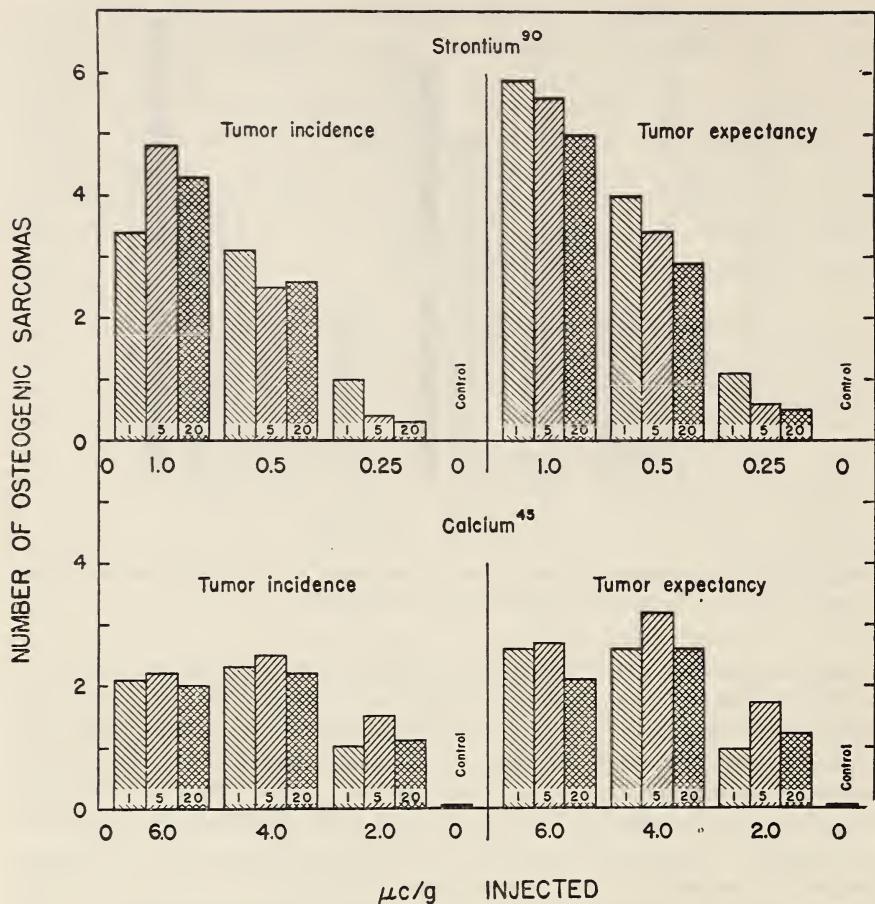


TEXT-Figure 2.—Average number of days lived after the first injection as a function of the total amount and the number of injections.

Tumor expectancy, comparable to life expectancy, is calculated by dividing the number of tumors still to appear by the number of animals still alive. Since tumor expectancy remained relatively constant from 150 days after injection until less than 3 animals remained alive, the average of the values obtained at 10-day intervals was used. The advantage of tumor expectancy over tumor incidence is well illustrated by these two experiments. In the Sr⁹⁰ experiment, the relationships among the groups are not the same when tumor incidence and tumor expectancy are considered. But in the Ca⁴⁵ experiment there is very little difference. Mice in both of these experiments were relatively free of infectious disease. The "incidental deaths" that reduced the population in the Sr⁹⁰ experiment and distorted tumor incidence were deaths due to lymphocytic neoplasms (3). These tumors were not increased in the Ca⁴⁵ experiment.

Tumor expectancy after Sr⁹⁰ administration decreased as the total amount injected decreased and also as the number of fractional injections increased. The same general relationships appear in text-figure 4, in which the rate of mortality with osteogenic sarcomas is plotted. The daily disease-specific mortality rate was derived from the rate calculated every 10 days for a 50-day period from the time of injection until less than 3 mice remained alive.

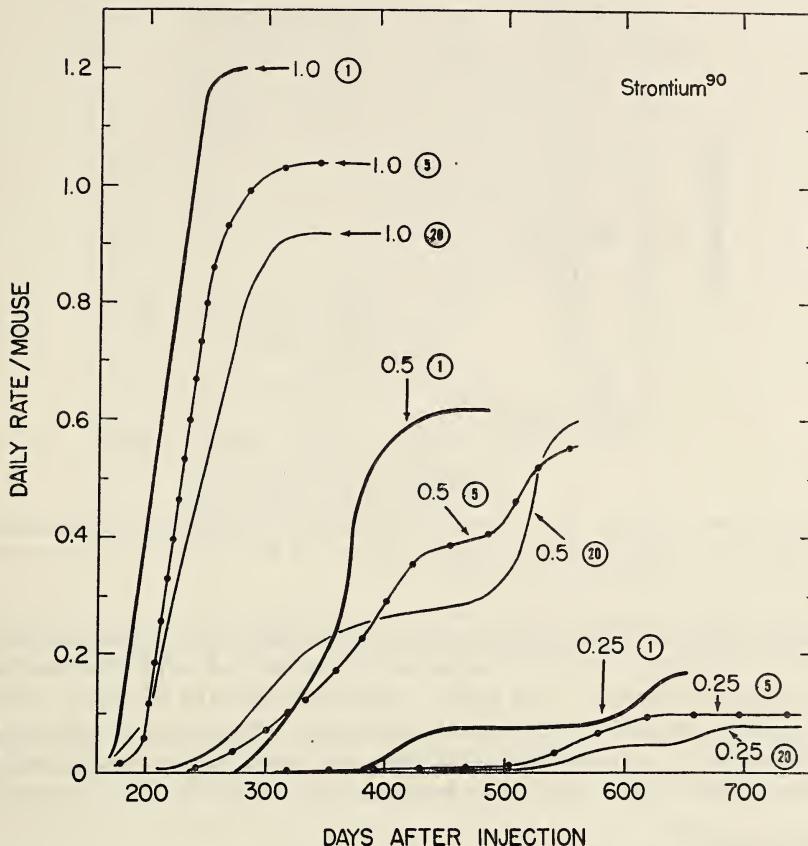
Tumor expectancy after Ca⁴⁵ administration did not follow a simple pattern (text-fig. 3). The carcinogenicity of 6.0 μ c per g was no greater than that of 4.0 μ c per g, and at each dosage level 5 injections resulted in more osteogenic sarcomas than either a single injection or 20. The disease-specific mortality rates in text-figure 5 show that 6.0 μ c per g actually was less carcinogenic than 4.0 μ c per g. Therefore, the data at



TEXT-FIGURE 3.—Incidence and expectancy of osteogenic sarcoma as a function of the total amount and number of injections. Incidence: total number of tumors per total number of mice. Average expectancy: number of tumors to come per number of living mice, calculated at 10-day intervals and averaged from 150 days after injection until less than 3 animals remained alive.

the highest level are not useful for evaluating the effect of fractionation. When the total dose was 4.0 or 2.0 μc per g, a single injection was much less effective than multiple injections, and 20 were less effective than 5.

Tumor expectancies are compared in another way in text-figure 6, which emphasizes the decrease in carcinogenicity in going from 1 to 5 to 20 fractions of Sr⁹⁰ and from 5 to 20 to 1 fraction of Ca⁴⁵, as well as the nonlinearity of the dose-response curve. There was 1 osteogenic sarcoma among the Ca⁴⁵ control mice, giving a tumor expectancy of 0.03. An extension of the line connecting 2 and 4 μc of Ca⁴⁵ per g injected in 5 fractions comes reasonably close to that value. However, extensions of each of the other lines transect the abscissa at values greater than zero. Reduction in dose level from 0.5 to 0.25 μc of Sr⁹⁰ per g resulted in an

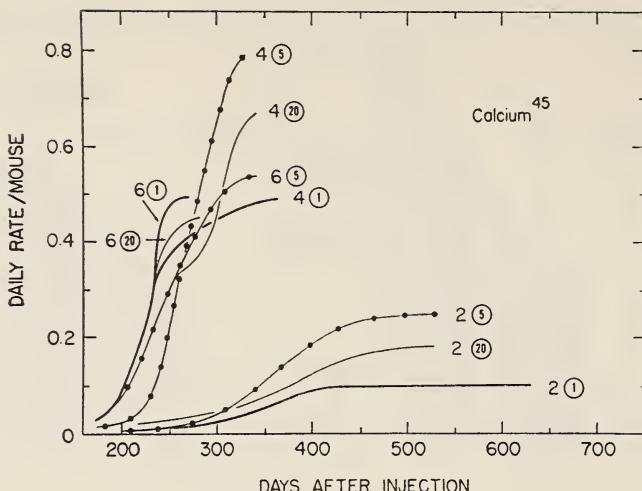


TEXT-FIGURE 4.—Daily rate of death with osteogenic sarcoma as a function of time after the first injection of Sr^{90} . Amounts of 1.0, 0.5, or 0.25 μc per g were given in 1, 5, or 20 injections.

average reduction in tumor expectancy to 1/5. Reduction in dose from 4.0 to 2.0 μc of Ca^{45} per g resulted in an average reduction in tumor expectancy to 1/2.3.

Discussion

Although the carcinogenicity of fractional exposure to Sr^{90} and Ca^{45} has been reported for rats (4, 5), the only study, apart from those presented here, comparing the response to a single injection and to multiple injections of the same total amount of a radionuclide is that with P^{32} reported by Blackett (6) and Lamerton (7). They found that 3 μc per g given in 1 injection produced 3 bone tumors in 20 rats, whereas 1 μc per g followed at 2-week intervals by 4 injections of 0.5 μc per g resulted in 19 tumors in 20 rats. This increase in response with fractionation was even more marked than the increase we observed with fractionation of Ca^{45} in mice. We obtained contrary results, however, with single and multiple injections of Sr^{90} .



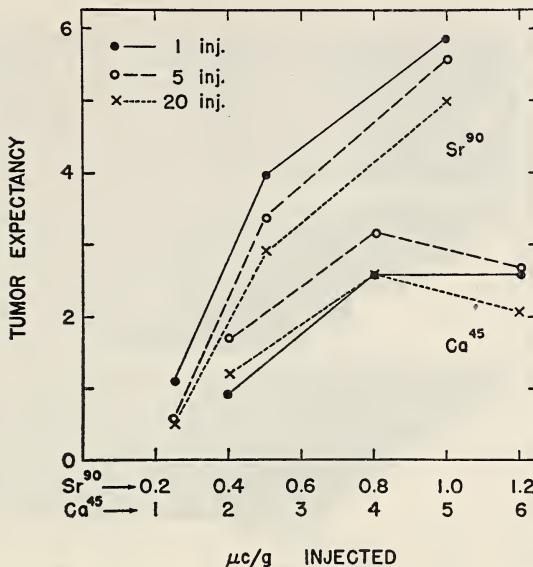
TEXT-FIGURE 5.—Daily rate of death with osteogenic sarcoma as a function of time after the first injection of Ca^{45} . Amounts of 6.0, 4.0, or 2.0 μc per g were given in 1, 5, or 20 injections.

The Sr^{90} and Ca^{45} experiments were comparable except that they were done at different times, the latter not being started until after the last Sr^{90} animal had died. It is highly improbable that the divergent results were due to subtle environmental variations or to genetic change in the mouse colony. A more likely explanation is based on the retention of the radionuclides, the range of the beta rays, and the rate of bone growth.

Strontium-90

The object of the Sr^{90} experiment was to examine the hypothesis that the initial high dose rate immediately after injection is largely responsible for the osteogenic sarcomas that appear many weeks later and that the dose accumulated from the retained radionuclide months later is negligible with respect to tumor induction. With fractionation, it is possible to deliver the same total dose as that given in a single injection and at the same time to limit the maximum body burden to 48 percent (5 fractions) or 41 percent (20 fractions). The body burdens of the animals receiving a single injection exceeded these levels for 5 and 8 days, respectively, and these mice contained more Sr^{90} than those receiving multiple injections during the first 18 days of the experiment. The average difference in the maximum burden during the injection period of the mice receiving 5 and 20 injections was about 10 percent.

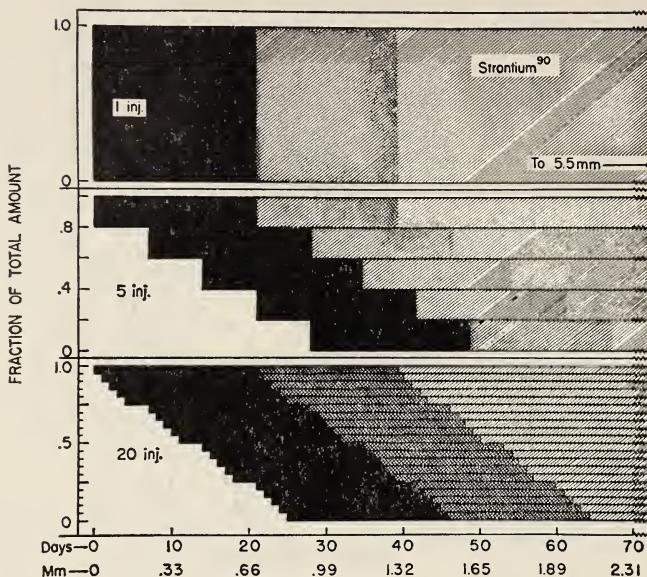
The actual deposition sites varied with the 3 regimens of injection of Sr^{90} - Y^{90} but this was not very important since the range in bone of the Sr^{90} - Y^{90} beta particles is long in relation to the size of mouse bones (text-fig. 7). Since the radiation cannot be shown in all dimensions, it is shown in only one. The injected material is represented as being deposited in the calcifying region proximal to the distal epiphyseal plate of the femur



TEXT-FIGURE 6.—Average expectancy of death with osteogenic sarcoma after 150 days as a function of amount injected. The Ca^{45} scale of μc per g is 5 times higher than the Sr^{90} scale.

because this bone provides useful reference points for evaluating the range of radiation (2). At 70 days of age the femur is about 14.6 mm long, the calcifying zone is about 0.3 mm wide, the diameter is less than 1.5 mm, and the cortex is about 0.12 mm thick. The femur is increasing in length at the rate of 33 μ per day, and most of the growth occurs at the distal epiphysis. The full-grown femur is about 16.3 mm long.

In view of the dimensions of mouse bone and the fact that the maximum range of the beta particles is 1.1 g per cm^2 and the dose they deliver is reduced to half by about $\frac{1}{3}$ mm of bone, the exact microscopic location of the Sr^{90} has little effect on the total volume of bone irradiated or upon dose intensity at any specific site. Therefore, the explanation for a single injection being more carcinogenic than 5, and 5 being more carcinogenic than 20 must lie in the temporal relationships of body burden or dose rate. A single injection was most effective because the dose rate exceeded that of the other injection schedules for 5 days, and 5 fractions were more effective than 20 because the maximum dose rate during the injection period was about 10 percent higher with the 5 fractions. Comparisons of tumor expectancy when 0.5 and 0.25 μc per g were given in 1 or in multiple injections indicate that the radiation received during the 1st week after a single injection affected the incidence of osteogenic sarcomas by about 36 percent. Also, the difference of 10 percent in maximum body burden during the period of injection of 5 or 20 fractions may have been responsible for the observed 16 percent difference in tumor expectancy.



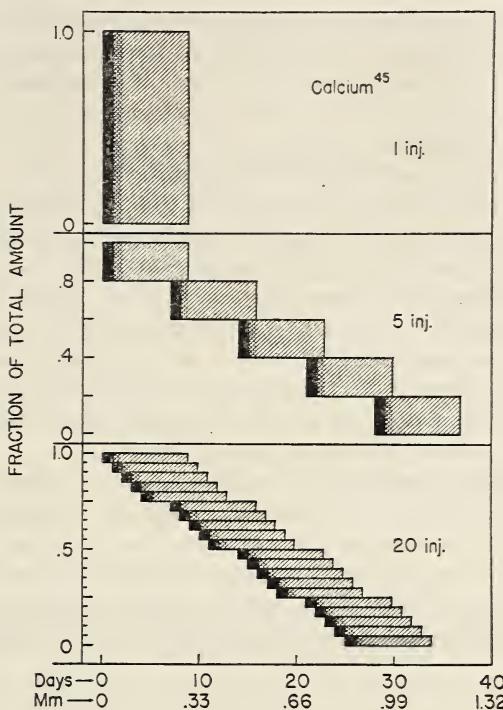
TEXT-FIGURE 7.—Proportion of total amount of Sr^{90} - Y^{90} in successive positions of the actively growing distal epiphyseal plate of the femur as a function of injection schedule and range of radiation. *Light shading*: maximum range, 5.5 mm. *Medium shading*: average distance, 1.3 mm. *Heavy shading*: distance at which the dose rate from a thick plane source in bone drops to $\frac{1}{2}$ (0.69 mm).

Calcium-45

The purpose of the duplicate experiment with Ca^{45} was to investigate carcinogenicity with a weak beta-emitter that had the same microscopic distribution as Sr^{90} . We had not anticipated that the greater retention of calcium would so greatly decrease the difference in body burden between single and repeated injections. Although the body burden after a single injection exceeded that after repeated injections for 6 days, the maximum difference was only 26 percent instead of the 52 percent observed when Sr^{90} had been given. Another disparity was that after the 4th day the maximum body burdens with 5 and 20 fractions were very similar.

The range of the Ca^{45} beta ray is short compared to the size of mouse bone, as illustrated in text-figure 8. Thus the spatial distribution of the dose depends on the microscopic location of the isotope, and the exact deposition site varied markedly with the three injection schedules. The osteogenic sarcoma data probably can be explained by the differences in both the spatial distribution and the temporal distribution of the absorbed dose.

Five fractional injections were more carcinogenic than 1 or 20, and 20 fractional injections were more carcinogenic than 1. A single injection resulted in the intense irradiation of a limited volume of bone. Since the dose rate was reduced to half in 36μ and the total range extended over only 290μ , the dose rate throughout the femur was very uneven. In



TEXT-FIGURE 8.—Proportion of total amount of Ca^{45} in successive positions of actively growing distal epiphyseal plate of the femur as a function of injection schedule and range of radiation. *Light shading:* maximum range, 0.29 mm. *Medium shading:* average distance, 0.06 mm. *Heavy shading:* distance at which dose rate from a thick plane source in bone drops to $\frac{1}{2}$ (0.036 mm).

addition, the femur was growing in length at the rate of about 33μ per day. The physiological causes of deposition of Ca^{45} in a particular location also caused the deposition of stable calcium in that location, with the result that the Ca^{45} deposit was soon buried in solid bone. It is doubtful that osteocytes give rise to osteogenic sarcomas, so that once Ca^{45} is buried it probably is not responsible for many bone tumors. Therefore, the effective carcinogenic life of the unevenly deposited Ca^{45} can be measured in days. This view is supported by experimental data and arguments in reports by Marshall and Finkel (2, 8) and Marshall (9).

Five fractional injections of Ca^{45} produced 5 times as many spots of concentrated radioactivity that were, on the average, $\frac{1}{5}$ as intense. Another difference was that cells adjacent to the calcified and calcifying bone were potentially exposed to radiation 28 days longer.

Twenty fractional injections could have produced 4 times as many hot spots as 5 injections, with an average concentration of Ca^{45} one-fourth as great if the interval between injections had been long enough. Since there were 5 injections each week during the injection period, and since growth at a specific site continues for more than 1 day, there was chance for the

Ca^{45} administered on successive days to be deposited in the same area of growth. As a consequence, the actual number of hot spots would be less, and their average intensity would be more. Nevertheless, with 20 injections a greater volume of tissue was irradiated, and the dose rate was more uniform throughout the bone. The actual exposure time with 20 fractions was 3 days shorter than the exposure time with 5 fractions.

These various factors lead us to suggest that dividing the total amount of Ca^{45} into 5 fractions produced more osteogenic sarcomas than a single injection because the volume of tissue irradiated was 5 times greater and the effective exposure time was 28 days longer. These conditions more than compensated for the fact that each hot spot contained, on the average, only $\frac{1}{5}$ as much Ca^{45} . In view of the repeated observation that the carcinogenic response to bone-seekers increases approximately as the square of the amount injected (8, 10), reduction of the dose to $\frac{1}{5}$ could be expected to reduce the number of tumors to $\frac{1}{25}$.

Twenty fractions were less efficient than 5, a result that can be explained by an appropriate balance between the increase in the volume of tissue irradiated and a decrease in the exposure time and the intensity of the hot spots. That 20 exposures were more efficient than 1 implies that the greater volume of tissue irradiated and the prolonged exposure more than compensated for the reduced intensity of the hot spots.

Phosphorus-32

The P^{32} experiment of Blackett (6) and Lamerton (7) introduced another variable that we have not yet tested adequately in mice, namely, the half-life of the radionuclide. Since the P^{32} beta ray has a maximum range of 4 mm in bone, the spatial distribution of the dose in mouse bone would not differ much from that described for $\text{Sr}^{90}\text{-Y}^{90}$. Rats were used in this study, so the dose was not as uniform throughout the bone as it would have been in the smaller mouse. A pertinent observation is that of Vaughan and Owen (1), who found that in the rabbit, which is much larger than the rat, the ratio of maximum to minimum dose rate in bone from injected Sr^{90} was about 40. An uneven distribution of the dose from P^{32} in the rat would favor an increase in carcinogenicity with fractionation because a larger volume of tissue is irradiated with multiple injections.

Still more important, however, is the fact that excretion reduces the amount of P^{32} in the rat to about 51 percent 14 days after administration, when about 62 percent of the retained P^{32} is in the skeleton (11). Physical decay in 14 days reduces the amount further, so that only 25.5 percent of the injected P^{32} remains. We have estimated that the body burden of the rats that received a single injection exceeded the body burden of the other animals until the second injection (day 14). After the third injection (day 28) the latter animals contained much more P^{32} than those that had received a single injection. The maximum difference occurred at the time of the last injection (day 54), when the body burden after a single injection was 2 percent and the rats receiving multiple injections contained

25 percent. Consequently, on the basis of body burden, or temporal distribution of the dose, more tumors should arise with fractional injections, and this indeed was true.

Continuous Exposure

Mice were maintained on a constant diet of Sr^{90} , with the result that the amount of Sr^{90} they contained increased gradually and the distribution of the absorbed dose both in space and time was as uniform as could be attained. The radionuclide was incorporated in the food in amounts ranging from 15 to 0.01 μc per g calcium according to the method described earlier (12).

The experiment was started with adult CF1 females that were mated 2 days after they were given Sr^{90} food. They and their offspring continued on the same food as long as they lived. The male offspring, except those on the 15 μc per g calcium food, were used for radioanalysis and autoradiography.

Results

The current osteogenic sarcoma data are summarized in table 1. One animal is still alive, and the histologic examinations are not completed.

Only the food containing the greatest concentration of Sr^{90} (15 μc per g calcium) unquestionably increased the incidence of osteogenic sarcomas among the mice that ate it first when they were about 150 days old. The number of tumors obtained when the food contained 2.5, 0.1, and 0.01 μc of Sr^{90} per g calcium was within the control range, and the absence

TABLE 1.— Sr^{90} ingestion: current osteogenic sarcoma data

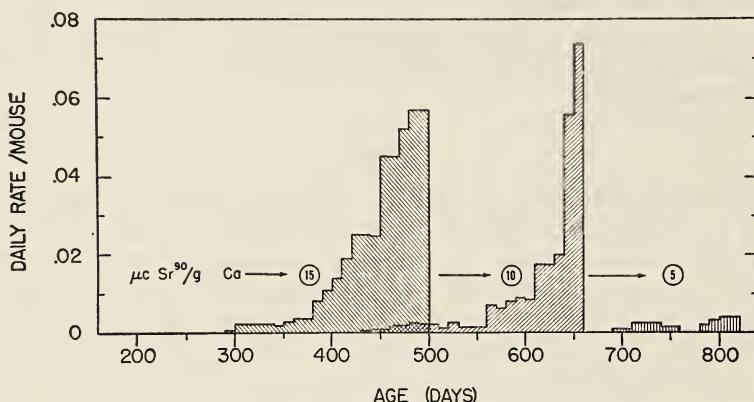
Initial age	Sex	μc of Sr^{90} per g Ca	Number of mice	Tumor incidence*	Tumor expectancy†
~ 150 days	♀	15	44	1. 07	1. 3
		10	41	0	0
		5	21	0	0
		2. 5	46	0. 043	0. 03
		1. 0	20	0	0
		0. 1	39	0. 026	0. 02
		0. 01	31	0. 032	0. 01
<i>In utero</i>	♀	0	46	0	0
		15	50	1. 26	1. 9
		10	95	0. 28	0. 71
		5	53	0. 06	0. 10
		2. 5	129	0. 008	0. 014
		1. 0	49	0	0
		0. 1	93	0	0
<i>In utero</i>	♂	0. 01	71	0	0
		0	146	0	0
		15	57	0. 35	0. 52
		0	45	0	0

*Tumors per mouse.

† Number of tumors to come divided by number of animals alive, calculated at 10-day intervals and averaged from the age of 150 days until less than 3 mice remained alive.

of tumors at intermediate levels supports the view that they were spontaneous rather than radiation-induced. The female mice first exposed to 15, 10, or 5 μ c food before birth had more tumors than normal, but the results with the 2.5 μ c food were within the control limits. The male mice that ate the 15 μ c food had only one fourth as many malignant bone tumors as their female littermates.

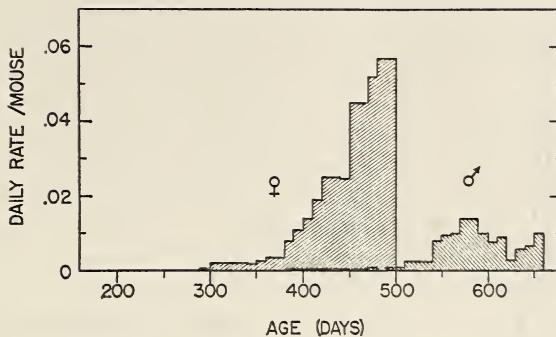
The mortality rates with osteogenic sarcoma of the female mice exposed to Sr⁹⁰ from the time of conception are shown in text-figure 9. The daily rate was derived from rates over a 50-day interval calculated every 10 days as long as 3 or more mice remained alive. The first tumor death occurred at 325 days when the dietary level was 15 μ c per g calcium, at 457 days when it was 10 μ c per g calcium, and at 717 days when it was 5 μ c per g calcium.



TEXT-FIGURE 9.—Daily rate of death with osteogenic sarcoma as a function of age among female mice that had always been maintained on food containing 15, 10, or 5 μ c of Sr⁹⁰ per g calcium.

The difference in response between the male and female littermates that ate food with the highest level of Sr⁹⁰ is illustrated in the mortality rate with osteogenic sarcoma in text-figure 10. The first tumor death among the males occurred 84 days later than in the females, but the rate among the females at that time was still low.

The difference in tumor response related to the age at which the Sr⁹⁰ was first ingested is shown in text-figure 11. The earliest tumor deaths occurred at about the same time among the dams and their female offspring, but the slopes of their disease-specific mortality rates were quite different. The tumor data from the standard long-term toxicity experiment with Sr⁹⁰ that are most similar to the consequences of eating food containing 15 μ c Sr⁹⁰ per g calcium are those resulting from the injection of 0.44 μ c per g (10, 18). Those data are included in text-figure 11, and the body burdens of the 3 groups of mice are compared in text-figure 12. The animals that had been exposed to Sr⁹⁰ since conception contained 2.3 μ c when they were 100 days old, and further increase was negligible. Their dams contained about 0.75 μ c after they had been eating Sr⁹⁰ for 100 days,

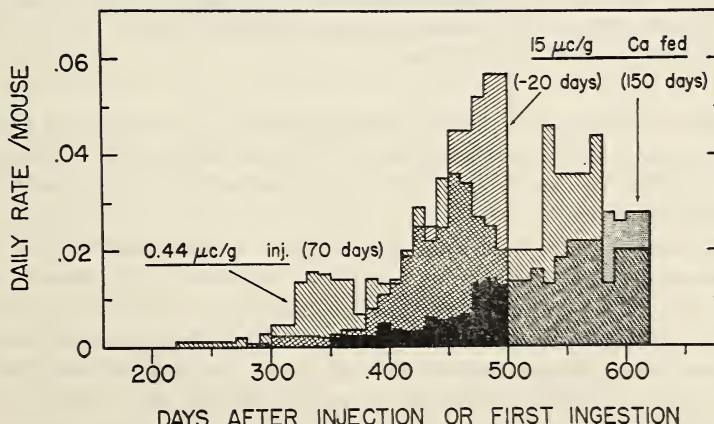


TEXT-FIGURE 10.—Daily rate of death with osteogenic sarcoma as a function of age among male and female mice that had always been maintained on food containing 15 μ c of Sr⁹⁰ per g calcium.

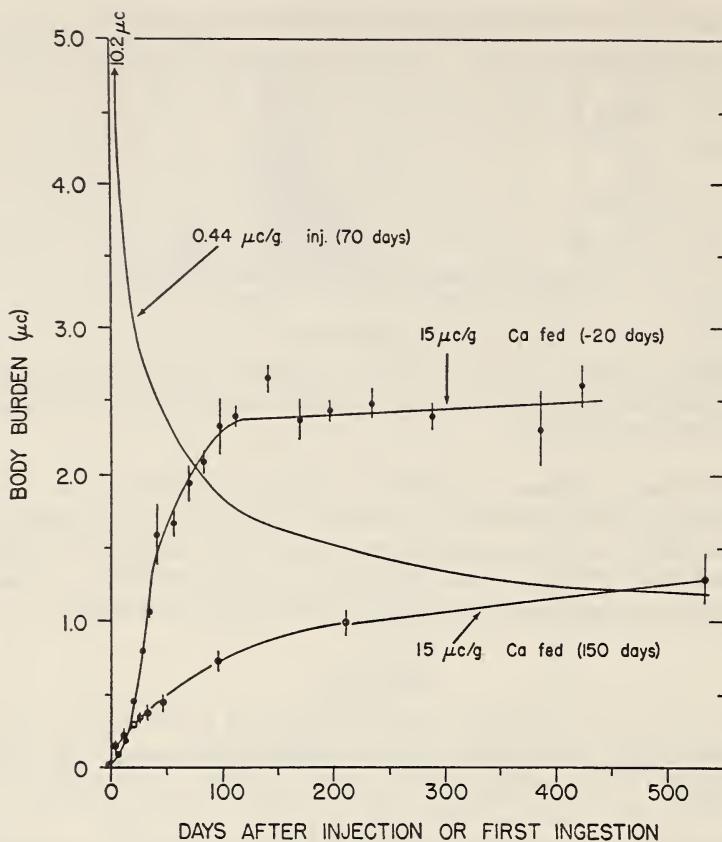
and their body burdens continued to increase gradually. In contrast, the animals that had received an injection of 10.2 μ c (0.44 μ c per g) when they were 70 days old contained 2.3 μ c 58 days later, and after 75 days they contained less Sr⁹⁰ than the mice on the continuous Sr⁹⁰ diet. However, their body burdens exceeded that of the older mice for 450 days.

Discussion

Tumor expectancy of the female mice exposed to Sr⁹⁰ since conception decreased as the concentration of Sr⁹⁰ in the diet decreased. As was true after injection, the carcinogenic response seemed to be related to the square, or perhaps to a somewhat higher power, of the dose. A decrease in dietary concentration from 15 to 10 μ c per g calcium (1/1.5) decreased



TEXT-FIGURE 11.—Daily rate of death of female mice with osteogenic sarcoma as a function of time after first exposure to Sr⁹⁰. One group received a single intravenous injection at 70 days of age, and the other two groups were maintained on food containing 15 μ c of Sr⁹⁰ per g calcium, one from conception and the other from about 150 days of age.



TEXT-FIGURE 12.—Sr⁹⁰ body burden as a function of time: 1) after injection of 0.44 μc per g (10.2 μc total) at 70 days of age; 2) after starting to eat Sr⁹⁰ at about 150 days of age; and 3) after birth when maintained on a continuous Sr⁹⁰ diet. Vertical lines denote limits of 1 standard error.

tumor expectancy to 1/2.7, and a decrease from 10 to 5 μc per g calcium (1/2) decreased tumor expectancy to 1/7. In the former, the carcinogenic responses differ by the 2.5 power of the dose, and in the latter by the 2.8 power of the dose.

The disease-specific mortality rates show a difference in the time of appearance of the osteogenic sarcomas associated with dietary level (text-fig. 9). If we assume that food containing 5 μc of Sr⁹⁰ per g calcium is close to the minimum concentration in the diet for obtaining measurable tumor response in the mouse, then the minimum carcinogenic body burden is about 0.8 μc . This level would be reached at 100, 50, and 25 days on the 5, 10, and 15 μc diets, respectively. Since these time differences are much smaller than the apparent 200-day difference in tumor time when the dietary level is 15 or 10 μc , dose rate alone is not the whole answer, and the contribution of at least part of the integral dose to tumor induction should be considered when suitable data are available.

The tumor response of male and female mice maintained continuously on food containing 15 μc of Sr⁹⁰ per g calcium is strikingly different (text-fig. 10). This was the first experiment in which we kept CF1 males for long-term study, and it demonstrates the importance of considering the possibility of sex differences in carcinogenesis. Individual caging will permit us to do further work with the pugnacious CF1 male mouse.

The difference in tumor response between the dams and their female offspring can be explained, at least partially, by their body burdens. Adult mice fed 10 μc of Sr⁹⁰ per g calcium would not reach the 0.8 μc level assumed above to be the minimum to give a measurable response for 420 days. It is not surprising, therefore, that there were no tumors among the adult mice on this diet because only 15 were alive at 420 days, and 12 of these died before 570 days, a time interval that would provide for the usual 150-day latent period between "induction" and death with osteogenic sarcoma. However, if this argument is valid and the dams can be compared in this fashion with their female offspring, then there were too many tumors among the dams fed 15 μc of Sr⁹⁰ per g calcium (table 1 and text-fig. 12). These confusing relationships will be studied further when the data are complete.

Equivalent carcinogenic amounts of injected and ingested Sr⁹⁰ were selected to compare the relative contribution of the initial high dose and the retained dose. The body burdens shown in text-figure 12 should provide the clue, but there are still too many unknowns. For that reason we have turned to experimental techniques that permit greater manipulation and control of the delivered dose.

Time-Limited Irradiation

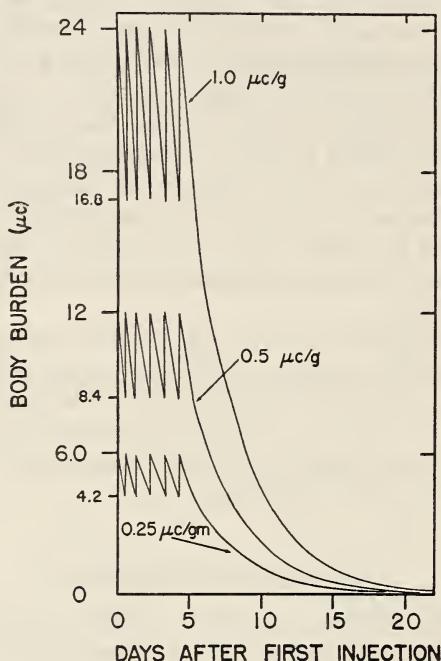
The bone-seeking radioisotopes are excellent carcinogens, but they present many obstacles to discovering why and how they induce osteogenic sarcomas. These obstacles derive from the fact that the dose delivered by radionuclides varies both in space and time, and most of them continue to irradiate the animal long after the neoplastic change has taken place. As a result, it has been extremely difficult to arrive at any reliable and universally acceptable estimate of the absorbed dose required for carcinogenesis. Two experiments in progress should simplify some of the problems related to residual body burden.

Yttrium-90

Y⁹⁰ is very useful for studying radiocarcinogenesis because: 1) It is deposited primarily in bone (14), 2) it emits an energetic beta ray, which, in fact, is largely responsible for the toxicity of Sr⁹⁰, and 3) it has a half-life of only 64 hours. We hoped that by maintaining various levels of Y⁹⁰ in the body for varying lengths of time we could separate and, thus, recognize the contributions of the dose rate and the accumulated dose.

The first step was to determine a carcinogenic amount of Y⁹⁰. In some earlier experience with Y⁹⁰, 10 to 12 μc per g given in a single injection

produced a few osteogenic sarcomas, but this amount also killed 50 percent of the mice within 30 days (15, 16). We assumed, accordingly, that fractionation would be more carcinogenic than a single injection. Seventy-day-old CF1 female mice were given repeated intravenous injections of Y^{90} according to a schedule that would maintain their body burdens for 5 days between 70 and 100 percent of 1.0, 0.5, and 0.25 μc per g, as shown in text-figure 13. Radioactive decay quickly eliminated the Y^{90} after the last injection.



TEXT-FIGURE 13.— Y^{90} body burden as a function of time after the first injection. Thirty percent of the initial amount was injected 11, 30, 52, 76, and 100 hours after the first injection.

The experimental routine includes weekly roentgenography of the bones of the living mice. This procedure permits the separation of the rate of appearance of osteogenic sarcomas from mortality rate. The data in table 2 include tumors in dead animals as well as tumors in animals alive at the moment. So far, the 0.5 μc per g maximum level is as carcinogenic as 1.0 μc per g, and the lowest injected dose level also has induced a few osteogenic sarcomas.

There is a striking difference between the early roentgenographic appearance and growth of these tumors and the tumors induced by Sr^{90} (17). The average time between the first roentgenographic recognition of an osteogenic sarcoma and death of the animal was 38½ days when Sr^{90} was used. The current average time for the Y^{90} osteogenic sarcomas is 170 days. The lesions seem to remain stationary for weeks, waiting

TABLE 2.—Y⁹⁰ injection: results at 590 days

Maximum body burden ($\mu\text{c/g}$)	Total amount injected (μc)	Number of mice	Percent mortality	Osteogenic sarcomas	
				Percent incidence	Tumors per mouse
1.0	60	30	53.3	13.3	0.13
0.5	30	60	48.3	13.3	0.15
0.25	15	120	45.0	4.2	0.05
0	0	120	52.5	0.8	0.008

for an additional stimulus before beginning progressive growth. This curious result introduces the possibility that a two-stage hypothesis of tumor production might apply to radiation-induced osteogenic sarcomas.

External Exposure

Exposure to X-rays is even more amenable to manipulation than exposure to Y⁹⁰. We first attempted to induce osteogenic sarcomas with X rays by exposing one hind leg of 70-day-old CF1 female mice to 2000 to 5000 r (18). Tumors appeared in all groups, but radiation damage was so severe that the carcinogenic response was impaired. In the next experiment smaller doses were given, and both hind legs were exposed to 250 KVP X rays at 58 to 59 r per minute.

The current results are given in table 3. Seven osteogenic sarcomas have appeared among the 750 mice that received 250 r. Of these tumors, 5 were in the irradiated area and 2 were not. Precise calculations have not been made, but a reasonable estimate is that the 250 r delivered about 500 rads to the bone.

The present data show an increase in tumors almost equal to the increase in dose. In fact, the data from this partially completed study indicate that the carcinogenic response (tumors per mouse) varies as the 1.13 power of the dose, a value that is almost linear. If this relationship continues to hold when all the data have been collected, it will provide a basis for examining the reason for the frequent observation that the incidence of osteogenic sarcomas increases as the square, or even the cube, of the injected amount of a bone-seeking radioisotope. The explanation should rest upon the fact that no isotope can deliver an absolutely uniform dose throughout the entire bone, and bone has three dimensions.

TABLE 3.—External exposure: results at 442 to 575 days

Exposure dose (r)	Number of mice	Percent mortality	Osteogenic sarcomas		
			Percent incidence	Tumors per mouse	Percent outside irradiated area
1,000	300	63.7	3.0	0.033	0.3
625	750	68.0	1.6	0.019	0.13
250	750	68.8	0.7	0.007	0.27

When an X-ray exposure dose is doubled, the absorbed dose is uniformly doubled. When the amount of radioisotope is doubled, the absorbed dose is doubled only in that volume of tissue that lies within the range of the emitted particles. The critical information still lacking concerns the definition and location of the region that must be irradiated if neoplastic change is to occur.

RESUMEN

Un gran cúmulo de información ha hecho posible predecir, con cierto grado de confianza, la respuesta carcinogénica de los ratones a los radioisótopos buscadores de hueso provisto que el animal en cuestión sea un ratón hembra CF₁ de 70 días de edad y que el material en prueba sea administrado en una sola inyección intravenosa. El trabajo presente describe cinco experimentos en curso, en varios estadios de terminación, en los que algunas de estas condiciones fueron alteradas.

Una inyección de Sr⁹⁰ y de Ca⁴⁵ se compara con 5 y con 20 inyecciones fraccionadas, así como también con una ingestión continua. La exposición continua a una dieta radioactiva desde el momento de la concepción es equivalente entre los ratones hembras y machos, y la exposición de hembras desde la concepción es comparable con una exposición que comenzará al rededor de los 150 días de edad.

La inyección fraccionada y la ingestión continua cambian el modelo de irradiación que sigue a una sola inyección proveyendo una distribución más uniforme de la dosis tanto en espacio como en tiempo. La aproximación opuesta es administrar una dosis limitada en espacio y en tiempo. El tiempo de limitación es estudiado usando Y⁹⁰, el cual tiene una vidamedia muy corta, y mediante la exposición solamente de las patas posteriores a dosis carcinogénicas de rayos X.

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PLATES

PLATE 13

FIGURE 1.—Autoradiographs of 50 to 80 μ sections of femurs arranged according to the amount of Sr^{90} injected, the number of fractional injections, and the time after the first injection.

$0.5\mu\text{c Sr}^{90}/\text{g}$ ONE
INJECTION

NUMBER OF INJECTIONS

1

5

20

5-8 23-27 62-63

AMOUNT INJECTED ($\mu\text{c Sr}^{90}/\text{g}$)

1.0

0.5

0.25

100

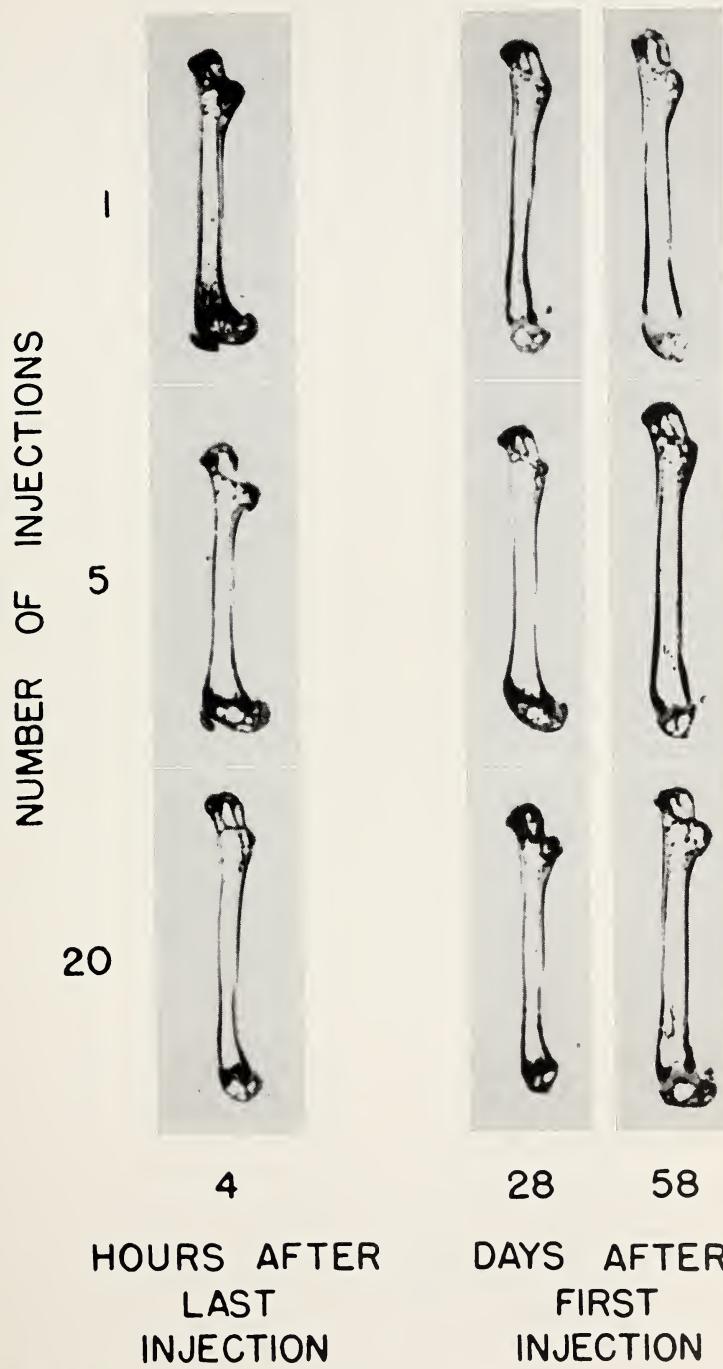
DAYS AFTER FIRST INJECTION

FINKEL, JINKINS, AND BISKIS

265

PLATE 14

FIGURE 2.—Autoradiographs of thick sections of femurs arranged according to the number of fractional injections of Ca^{45} and time after injection. The total amount was 6 μc per g.

$6\mu\text{c } \text{Ca}^{45}/\text{g}$ 

DISCUSSION

Lamerton: I agree entirely with your interpretation of the fractionation effect. If P^{32} is given to a growing bone in fractionated doses, a much larger part of bone is irradiated than with a single dose, and histologically there is a different sort of injury. With the dose of P^{32} that we were using, we saw gross damage extending over a much larger area after repeated injections than after a single injection. We have done some experiments using external irradiation, but not with the number of animals you have used, to determine the effects of fractionation, comparing repeated doses of 500 r with a single dose. There appears to be little effect of fractionation; if anything, I think the results show that with fractionated irradiation the tumor yield is a little lower. This is interesting in view of the effect of fractionation on radiation-induced thymoma incidence, as demonstrated by Dr. Kaplan. The presence of such a fractionation effect might depend on the involvement of virus factors in the carcinogenic process.

Dr. Finkel was rather scathing about using males, because they always fought. Our rats do not!

As to the conversion from roentgens to rads used by Dr. Finkel, I would hesitate about regarding 250 r in bone as equivalent to 500 r. The conversion factor depends entirely on how far from the bone surface are the cells that are responsible for bone tumor formation.

Finkel: I did not mean to imply that all males fight, but unfortunately, the CFL male does. This strain was available in 1945 when we needed several thousand at a time, and most other strains were not available in this quantity. We have also been using CBA mice because they were recommended by Dr. Mole, and because CBA males live together very well. However, we have not yet put CBA males into this kind of experiment.

We are very interested, of course, in knowing just what the dose in rads to the bone actually was in our X-ray experiment. The dose has not been accurately calculated at this time, but a physicist's guess was that the 250 r exposure dose probably corresponded to about 500 rads.

Kaplan: Some very interesting comments can be made on Finkel's presentation. One point she mentioned earlier was that one end of the bone is responsible for virtually all growth after a certain age. One wonders whether the tumors are localized in the growing end, the nongrowing end, or randomly distributed along the bone. Now that you can produce tumors in any bone you wish by external X rays, it seems to me that the growth rate of various bones could be investigated differentially by irradiating one side of a mouse longitudinally with a given dose, and using the other side of the mouse as its own control. I would expect that if the growth rate itself has anything to do with the carcinogenic process, the tumor incidence per bone would be a direct reflection of growth rate. Furthermore, it seems to me that the ability to localize the radiation over a particular bone would also lead to grafting experiments. It would be possible to put irradiated bones into nonirradiated sites which have good vascular supply and, conversely, to put nonirradiated bones into irradiated sites. Furthermore, it would be possible either before or after irradiation to subject locally irradiated bones to various kinds of hormonal and traumatic stimuli. I would be particularly interested in knowing what deliberate fractures would do to the tumor induction rate at a particular irradiated site. One might have difficulty in getting a mouse growth hormone and not a foreign species because of antibody problems. I have one minor question regarding the cumulative curves for 1, 5, and 20 injections of calcium. You discarded some data having to do with 6 μ c per g because you said it was too toxic. However, the single dose at 4 μ c per g broke off in exactly the same way as the 6 μ c per g. Therefore, it seems to me that if it were valid to omit the 6 μ c per g data, you ought also to discard the single-injection data at 4 μ c per g.

Finkel: We did not discard any data, but the results with 6 μ c Ca^{45} per g may not be very reliable because there is evidence that the radiation was too great. In our studies on the induction of osteogenic sarcomas, we prefer to use dose levels that yield

a good carcinogenic response but do not produce too many other changes that may complicate the picture. Because both 6 and 4 μ c per g severely decreased life expectancy, and because the tumor response was smaller with 6 μ c per g than with 4 μ c per g, it appeared that 6 μ c per g had done more than just induce tumors; it probably had produced extensive bone injury. For the same reason, though evidence for excessive bone injury is not nearly as great, we do not have complete confidence in the carcinogenicity data when 1 μ c Sr⁹⁰ per g was given in one injection. Actually, interpretation of the dose-response relationship is simpler if that particular value is omitted. The curve to which you referred was disease-specific mortality rate, not incidence. But that makes little difference in the argument, which would apply in either case.

You have suggested many interesting experiments. As for irradiating bone and putting it in a neutral site, or irradiating an area and putting a transplanted bone into it, we did just that a few years ago, but we did not get very far. Nevertheless, there are great possibilities in the approach, and I hope to return to these experiments.

You wondered if the tumors are localized in the growing area of the bone, in the nongrowing end, or if they are randomly distributed. When we inject a radioisotope, or specifically, when we inject Sr⁹⁰ into a 70-day-old CF1 female mouse, about 41 percent of the osteogenic sarcomas occur in the distal femur or proximal tibia. These are the areas where the isotope is concentrated because, in the 70-day-old mouse, the epiphyses at the knee are not closed. In the current experiment in which the hind legs were exposed to X rays, and the total femur was always included, most of the tumors have appeared in the proximal tibia, the same place that we would expect to see them if a bone-seeking isotope had been used. Some have occurred in the femur, and in the proximal rather than the distal end. The distribution so far seems to be a little different, but the impression is that the tumors arise in the growing areas.

Upton: I was interested to learn about the different growth rate of the yttrium tumors as a class, and I wondered if these tumors arose in the same site and if earlier in their development you examined them to see whether or not they are histologically quite different from the others. Is it conceivable that yttrium is complexing or chelating in some way to make these tumors antigenic as a class?

Finkel: The amount of yttrium that provides the radiation we need is actually very small; that is, the number of atoms of yttrium is so few that I doubt whether chelation or antigenicity could be factors. As for looking at some of the lesions that appear to be early tumors to see if they are histologically different, we cannot do that. There are not many animals in the experiment, the initial number having been determined as necessary to provide valid results for the three dose levels being studied. We have considered dividing the remaining animals and treating a portion of them with urethan, another dose of yttrium, or a dose of X ray. We have discussed these possibilities many times, and have decided to leave this experiment just as it is and start another one. Of course, a histopathologic study is being done, but only after the mice reach the end of their natural lives. Some of this material will be ready for study soon. The terminal lesions often are fine osteogenic sarcomas, as seen roentgenographically. Some of the early lesions start to grow after a long period, and ultimately they look like typical osteogenic sarcomas. They may even kill their host eventually. Quite a few, however, remain quiescent.

Mole: Dr. Finkel, surely the only difference between the Y⁹⁰ and the Sr⁹⁰ animals is that the latter continues to be irradiated.

Finkel: I think that the continuing irradiation is the only difference. With Sr⁹⁰ there is always a residual body burden, and probably the initial carcinogenic stimulus receives further impetus from it. With Y⁹⁰ the body burden is soon lost completely. In the X-ray experiment, however, where the irradiation is received in a short time and there is no body burden, osteogenic sarcomas are appearing. In that experiment we are not getting weekly roentgenograms of the animals, so I can say nothing about progressive changes in the skeleton. The terminal roentgenograms, however, have not shown any strange bone lesions or peculiar osteogenic sarcomas.

Kaplan: How difficult, technically, would it be to effect chromosome preparations on these bone tumors? I think it might be very interesting to compare the chromosomal picture in the yttrium tumors, particularly at an early stage, and possibly serially, with that in the strontium tumors. Although there is still a good deal of controversy, some people believe that the aneuploidy observed in many tumors is more closely associated with the evolution of autonomy in the tumor than with the initial neoplastic change. Perhaps the strontium tumors acquire a higher degree of aneuploidy much earlier in their evolution.

Finkel: That is a very good idea, if only we can figure out how to do it! I should like to add that this is the first time in our experience with various radioisotopes that we have come across a tumor with particular characteristics such that we can say what material probably induced it when we look at serial roentgenograms. We have never been able to tell, by looking at a roentgenogram, whether a tumor had been induced by radium, plutonium, P^{32} , polonium, or what have you. I have no idea why the Y^{90} tumors are different, and maybe the difference will disappear when all the data have been collected and all the material has been studied.

Court Brown: Could I ask about the consistency of these tumors? Are they soft or hard? If they are soft and the cells easily dispersed, then it would probably be possible to do cytogenetic studies on direct preparations without an intervening culture period.

Finkel: They are about as dense as the tumors induced by the other isotopes, but the general roentgenographic appearance of the tumor is somewhat different. I do not think the terminal tumors are any more apt to be more or less densely calcified than other osteogenic sarcomas. There is quite a variety among murine osteogenic sarcomas, and most tumors contain large areas that are not calcified.

Lamerton: It was not quite clear, Dr. Finkel, whether you were basing your views on the difference in tumor types produced by the strontium and yttrium solely on the evidence of radiographic examination. Are you sure that you were always looking at tumors? One can have considerable radiographic changes without malignancy.

Finkel: Most of the Y^{90} material still exists in living animals. A few mice have died, and the histologic work is being done. The differences I have been talking about are those that appear on the roentgenograms. Time after time in Sr^{90} animals we have seen lesions appear, progress, and, within a few weeks, develop into typical osteogenic sarcomas. Typical osteogenic sarcomas have many different faces, but there is a group of malignant bone tumors that is nevertheless typical. In the Y^{90} animals, roentgenographically similar lesions appear. However, they progress very slowly, and frequently they seem to be quiescent for many weeks. Eventually, some of the lesions have progressed into a rapidly growing tumor, which then has the appearance of an osteogenic sarcoma. During the quiescent period, which may be as long as 100 to 170 days, the lesions do not change, or there is sometimes even some slight evidence of repair. The original irregularity in the area may disappear, and the previous impression of impending malignancy may be lost.

When the lesions first appear on the serial roentgenograms, they cannot be diagnosed roentgenographically as osteogenic sarcomas. However, if they are in an animal bearing Sr^{90} , we know from experience that they probably will develop into unquestionable neoplasms within a few weeks. Yet, if they are in Y^{90} animals, the lesions persist with little or no change for many weeks. When the histopathologic work is done, we should know whether or not these quiescent lesions are neoplastic.

Cancer Production by Chronic Exposure to Penetrating Gamma Irradiation¹

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SUMMARY

Over-all tumor incidence may be a misleading index of carcinogenicity. Age-specific mortality or incidence rate can be much more revealing. In experiments in which female CBA mice were exposed to fractionated whole-body γ -radiation over a 4-week period to total doses of 1000 or 2000 r, the leukemogenic effect appeared early and was exhausted within 2 years. In the same experiment radiation seemed to act differently in the induction of other kinds of tumor, malignant or benign. The age-specific mortality rates for these lesions appeared to be increased in a constant proportion at all ages, *i.e.*, radiation had a multiplicative action. There was no or very little reduction in this carcinogenic action even in advanced old age and hence no exhaustion of effect with time. This

experimental difference between leukemia and other forms of neoplasia is mirrored in human experience. These results can perhaps be understood on the hypothesis that carcinogenesis depends on two successive cellular events, each of which can occur spontaneously and can be caused by radiation, with the additional assumption that the relative probabilities of these events are different for leukemia and for the other kinds of tumors. Any adequate hypothesis for carcinogenesis must explain how an increase in amount of carcinogenic exposure (radiation) can fail to lead to a further increase in tumor rate. Both leukemia and the other kinds of tumors show this saturation type of response.—Nat Cancer Inst Monogr 14: 271-290, 1964.

IF EXPERIMENTS on carcinogenesis by radiation are to be considered not from the point of view of the toxicity of radiation but to see if they provide clues as to the nature of cancer, it may seem simpler *a priori* to analyze the results of experiments involving single exposures lasting a few minutes rather than results of experiments involving multiple exposures spread out over a period of time. However, one central fact about experimental radiation carcinogenesis is that it seems much easier to produce murine leukemia with multiple exposures than with single ones. One possible explanation is simply that, when the exposure is protracted, more radiation gets into an animal without killing it than when single doses are given. However, even when the total dose is the same, protracted irradiation

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tion may be more leukemogenic than a single dose. An alternative view is that multiple doses are basically more carcinogenic. This view is in harmony with the idea that the appearance of an overt tumor depends on the occurrence of not just one but of two or more separate events in a cell or tissue focus (1, 2), ideas which have been discussed elsewhere in relation to radiation carcinogenesis (3).

ASSESSMENT OF TUMOR INDUCTION

Although it is commonly agreed that the main somatic hazard from chronic irradiation at relatively low daily doses is indeed carcinogenesis, the experimental evidence is not really abundant. The first systematic experimental studies were started about 20 years ago. The most thoroughly reported were those of Egon Lorenz and his collaborators. Table 1 lists the observed incidence of some categories of tumor in LAF₁ mice that were irradiated by γ rays every day from about 60 days of age until they were dead (4). In general, there seems to have been a radiation-induced increase in each of the kinds of tumor, but in detail the results appear rather puzzling. If as little as 110 r of total dose could induce a 25 percent incidence in mammary sarcoma, why was this induced incidence unchanged when the total dose was increased 20-fold? In fact, none of the tumor categories showed a progressive rise with increase in level of daily dose or in total accumulated dose, a result that must throw some doubt on whether the radiation exposure was really responsible for the extra tumors. The numbers of animals were, of course, sufficiently small for each of the observed percentages to have a large coefficient of variation.

There is another factor, however, which must be considered. The survival times of the mice were progressively shortened as the daily dose was increased and, if tumors occur late in life because time is required for their induction and subsequent growth to the size at which they can be recognized, then the observed over-all tumor incidence may have been, so to say, artificially reduced by the life-shortening processes. When the data for pulmonary tumors are presented in a different way, as tumor

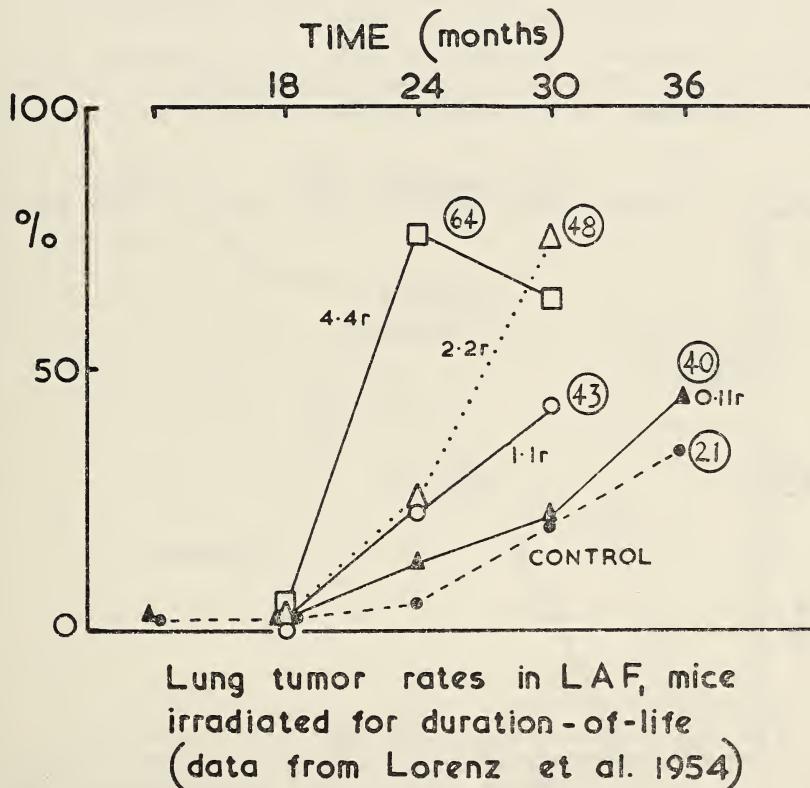
TABLE 1.—Incidence of some categories of tumor in chronically irradiated LAF₁ mice (4)*

Daily dose (r)	0	0.11	1.1	2.2	4.4
Accumulated dose (r)	0	110	780	1400	2600
Percentage incidence of tumors					
Mammary carcinoma	0	0	4	15	8
Mammary sarcoma	0	25	25	25	21
Lung tumors	21	40	43	48	64
Hepatoma	5	3	10	14	11

*The information from the exposures to the daily dose of 8.8 r is not included since there are internal discrepancies within and between the various tables in the original reference.

appearance rates (text-fig. 1), the results may appear to be much more consistent and meaningful. A progressive increase in the level of daily dose is now seen to have produced a progressively increasing effect and the induced tumor rate at the lowest daily dose is hardly distinguishable, instead of amounting to a doubling of the control level (table 1). It is clear that the way results are presented can appear to make a great difference to their meaning, as was also the case in our own experiments on daily irradiation by fast neutrons (5) and, indeed, also in experiments on the carcinogenic effects of single radiation exposures (6).

LAF₁ mice have a high natural incidence of leukemia, about 20 percent in males and 40 percent in females in Lorenz's experiments. With these mice it was concluded that a significant increase in incidence was obtained only with a sufficiently high daily dose and a large total dose. The effect of the 8.8 r daily level of exposure was not quite to double the natural incidence even though the total accumulated dose was over 4000 r. How-

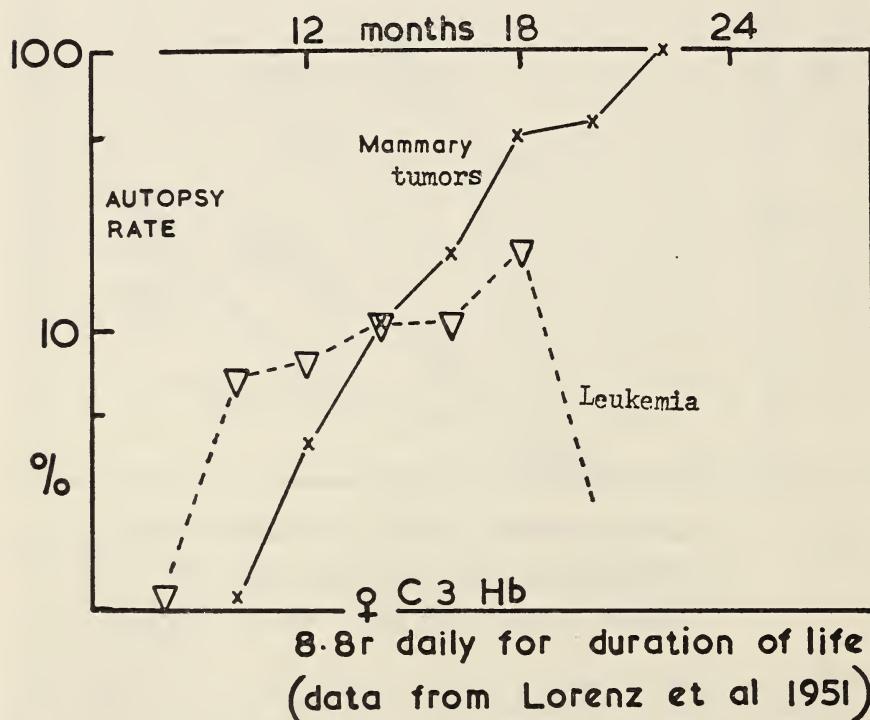


TEXT-FIGURE 1.—Lung tumor rates in LAF₁ mice irradiated for the duration of life. The data are taken from table 3.8 of (4). Lung tumor rates were calculated for successive nonoverlapping 6-month periods as the ratio (percent) of number of mice dying with lung tumors during a 6-month period to number of mice alive at the beginning of that period. The level of daily dose is shown. Numbers in circles are the over-all lung tumor incidences (table 1).

ever, when a different strain of mouse was used, C3Hb, which has a natural leukemia incidence of only a few percent, the same daily exposure increased leukemia incidence to 36 percent (7).

One very important question is whether continuing radiation exposure leads to a progressively increasing rate of induction of cancer. The leukemia data for C3Hb mice and data for their mammary tumors are plotted in text-figure 2. These two forms of neoplasia clearly behave differently. The rate of appearance of mammary tumors increased progressively and almost linearly with time (age and/or exposure), whereas the rate of appearance of leukemia increased slowly and then failed altogether in old age. This difference in behavior of leukemia and other kinds of tumor is found in CBA mice too, another strain in which there is a relatively low spontaneous incidence of leukemia-like disease and malignant tumors.

It is certainly much easier to demonstrate the statistical significance of an increase in tumor incidence of, say, 30 percent when the increase is from 1 to 31 percent than when it is from 30 to 60 percent, but it also seems to be true that strains of animals with a high spontaneous incidence are not for that reason necessarily unusually susceptible to tumor induction by radiation. In C3H mice carrying the mammary tumor agent



TEXT-FIGURE 2.—Leukemia and mammary tumors in female C3Hb mice receiving 8.8 r daily for the duration of life (4). Autopsy rates were calculated for successive nonoverlapping 2-month periods (as before).

and, therefore, with a high natural incidence of mammary tumors, exposures to daily irradiation did not lead to any obvious change in incidence (4), unlike C3Hb mice (text-fig. 2) that did not carry the agent and had a low natural incidence. However, the radiation-induced tumors in the agent-free mice appeared later in life than the naturally occurring tumors in mice carrying the agent. In mice with the agent the inevitable loss of tumor-carrying animals from the experiment, before the radiation-induced tumors could be expected, may have completely obscured the effect of the radiation exposure. Again, time itself has become a parameter that one must consider when interpreting experimental results, and it seems necessary to conclude that some experiments must be done on a large enough scale for rates of tumor appearance to be calculable, not just over-all incidence.

LEUKEMOGENESIS BY RADIATION IN CBA MICE

Similar problems of interpretation arise in our own experiments in which CBA mice were given daily irradiation by equal daily doses for varying periods of a few weeks to a few months (*cf* 8) but, nevertheless, a few interesting conclusions can be drawn. First, there was clearly a sharply sigmoid dose-response curve (9). This seems to be a general finding for leukemogenesis by single doses as well as by multiple doses and has an important bearing on the interpretation of a great deal of work on the various factors, such as endocrine influences, which can be shown to affect leukemia incidence (10, 11). If a comparison is made using only one particular dose and pattern of radiation exposure, then a large difference in percentage incidence of leukemia may or may not mean more than a trivial change in the leukemogenic potency of the radiation (12).

The second interesting finding was that there was a saturation effect for the development of leukemia. At some levels of daily dose a leukemia incidence of about 50 percent was regularly induced, but prolongation of exposure and therefore increase in total dose did not increase further the incidence, although other patterns of daily exposure to X rays could raise the incidence to around 100 percent (9, 13). In the first 20 months of life the female CBA mouse at Harwell has a leukemia incidence of less than 1 percent.

Thirdly, there was a strikingly different leukemia incidence after daily exposure to X rays and γ rays. Further experiments suggested that this was attributable to the difference in instantaneous dose rate of the actual exposures (13, 14). In these experiments the average dose rate over the whole exposure time was kept constant and also the number of daily fractions into which the dose was divided. The instantaneous dose rate at which a daily dose was given varied systematically between the experimental groups with a corresponding variation in leukemia incidence. The mice were randomized between the different treatment groups before the exposures started, and in each of the three experimental replicates all

the exposures started on the same day and finished together. The experimental design was intended to eliminate any complications in interpretation owing to possible differences in radiosensitivity with changing age or between different batches of mice.

At the time these experiments were started it was still generally accepted that the radiation-induced mutation rate for "point" mutations was independent of dose rate and depended merely on total dose. On this basis the leukemia results seemed to show conclusively that leukemia induction must depend on more than just a single mutation-like event. However, that conclusion is now much less clear-cut in the light of the recent evidence that the mutation rate in mammalian spermatogonia and oocytes may vary (within limits) with dose rate (15). Moreover, the observed change in leukemia incidence was as sharply and systematically correlated with the radiation-free interval between the fractions as with the instantaneous dose rate, since the lower the dose rate the longer the duration of a fraction and, therefore, the shorter the radiation-free interval before the next fraction begins. The relative importance of these two factors was the next object of study.

EXPERIMENTAL PROCEDURE

Female CBA mice were used at the same starting age as before and the whole of the radiation exposure from cobalt-60 γ rays was given, as before, within the limits of a 4-week period after the first dose. Mice were given their dose either in 672 successive hourly fractions for 28 days, in 112 fractions given 6 hourly for 28 days, in daily exposures 5 days a week for 4 successive weeks, in 14 fractions given every other day, in 7 fractions given every 4th day, or in 4 fractions given every 7th day. At the instantaneous dose rate of 0.3 r per minute and a total dose of 1000 r the size of the individual fractions was 1.5, 9, 50, 71, 143, and 250 r, respectively. Other groups of mice received total doses of 500 or 2000 r at the same instantaneous dose rate, and in the same patterns of fractionation, the size of the individual fractions being respectively half or double that listed for the 1000 r mice. A fourth set of mice received a total dose of 1000 r in the same patterns of fractionation and with the same size of fraction as those listed (omitting the hourly and 6 hourly groups) but at a higher instantaneous dose rate of 1 r per minute. One additional group of mice received 2000 r in 20 fractions 5 days a week at this higher dose rate. It was hoped that this experimental plan would provide information on the relative importance of the following factors: instantaneous dose rate, size of individual fraction, interval between successive fractions, total exposure dose. Since one radiation source was used for all the exposures to ensure constant geometry and conditions of exposure, the 30 experimental groups could not all be drawn in a random manner from one and the same starting population. This inevitably detracts from comparisons between different groups, but control mice were maintained

from each batch used. There were 21 different batches of animals, the first exposure being in March, 1957, the last in April, 1961. Not all the mice are dead and complete results from the 500 r exposures are not yet available. The succeeding analysis is certainly incomplete and may need revision in detail.

Cages were examined daily, including weekends, for dead mice which were autopsied systematically. Tissues were routinely taken to confirm any macroscopic diagnosis of a cause of death, except when this was due to infarction of, or hemorrhage from, a large hepatoma or to hemorrhage into an ovarian tumor or cyst in which the chances of finding tissue that could be diagnosed microscopically proved very small. Bone marrow was examined microscopically in almost every case. In addition a weekly inspection of every living mouse made it possible to kill those that were clearly going to die soon with gross lesions. From practically all such mice, a white cell count of heart's blood was taken immediately after killing. No mouse has been excluded from the subsequent analysis, however great the degree of postmortem decomposition.

RESULTS

A sharp distinction has been drawn between incidence of tumors and causes of death. When considering tumor incidence, each tumor was counted separately, regardless of what other tumors might be present in the animal; when mortality was being considered each mouse was counted only once. If there was more than one cause of death, *e.g.*, abdominal hemorrhage from an ovarian tumor in a mouse subsequently found on microscopic examination of the bone marrow to have gross malignant disease of reticular tissue, then it was counted as contributing one half to this latter category and one half to the category of lethal benign tumors. Cancers and nonreticular sarcomas have been grouped together: Either evidence of distant metastases or microscopic evidence of invasion was a necessary part of either diagnosis. Without one of these features tumors were counted as benign whatever their cellular appearance and whether they were clearly lethal or not.

Leukemia Incidence

During the first 15 months of observation practically every death was due to some form of malignant disease of reticular tissue, commonly lymphoblastic leukemia with a large mediastinal mass. However, in a fair proportion, the thymus was not involved grossly or the cell type was different. In a number, the diagnosis would have been impossible without microscopy of the bone marrow since there was nothing abnormal to the naked eye. For the present purpose all forms of malignant disease of reticular tissue have been included under the term leukemia, including all cases of gross aplasia of the bone marrow whether or not "leukemia" cells were identified.

Some of the results have been illustrated and discussed elsewhere (12, 13). At an instantaneous dose rate of approximately 0.3 r per minute, the optimum fractionation for producing leukemia was quite different for total doses of 1000 and 2000 r. After a total dose of 1000 r, leukemia incidence seemed to increase with increase in instantaneous dose rate for all fractionation patterns tried, *e.g.*, whether the radiation-free interval was 1, 2, 4, or 7 days. Nevertheless, the whole pattern is complex and does not seem susceptible of a simple, unifying interpretation.

With daily irradiation at the instantaneous dose rate of approximately 1 r per minute, the leukemia incidence after 2000 r was actually less than after 1000 r even though the 2000 r mice lived longer. After 2000 r the development of typical mediastinal lymphoblastic leukemia was delayed about 9 months, as compared with the standard development pattern shown by the 1000 r animals and by all our other work with X rays (9). It is difficult to see how this exceptional behavior can be accounted for except through some action of irradiation on the host. The dose of 2000 r given in this way killed acutely about one third of the mice. If it is accepted that host factors concerned in the development of leukemia can be modified by dose rate and fractionation, then a simple explanation for the experimental results is not to be expected.

Tumor Production

Since murine leukemia kills relatively early in life, the leukemia incidence in an experimental group determines the number of mice in which more slowly growing tumors can be detected. When there are very different incidences of leukemia in various experimental groups, it may be quite misleading to express tumor incidence as a fraction of the initial number of animals in the group. One possible way to circumvent this problem is to choose some moment when all or most of the leukemia has occurred and to consider tumor incidence only in the mice alive at this time and dying subsequently. This is illustrated in table 2, where each mouse has been counted only once. The numbers suggest some real effect of radiation in increasing cancer incidence, no effect on "leukemia," and a real reduction in animals dying from nontumorous causes. However, the data

TABLE 2.—Deaths by cause in mice alive at the beginning of the 19th month after the start of irradiation

Groups	Number of mice at risk	Malignant disease of reticular tissue (hemato-sarcomas) (%)	Other cancers and sarcomas (%)	Lethal benign tumors (%)	Causes other than tumors including "no cause identified" (%)
Unirradiated controls	274	11	14	41	34
0.3 r per minute	{ 1000 r 134 2000 r 86	8	21	59	12
All groups combined		12	24	56	8

seem much more revealing when analyzed according to mortality or incidence rates with time.

Mortality Rates in Unirradiated Female CBA Mice

The mortality rate has been calculated over 3-month periods as the number of mice dying from some cause during that period divided by the number alive at the beginning of the period. Text-figure 3 gives for control mice the over-all mortality rate and the rates for deaths from malignant disease of reticular tissue, from all other tumors, and from nontumorous causes. These latter included such grossly macroscopic categories as intestinal obstruction from bands of undetermined origin or from intussusception of the colon, but the majority belong to the category of undefined causes, including renal disease. The mortality rates are plotted on a logarithmic scale, not because any significance is attached to Gompertzian ideas but simply for convenience of subsequent discussion.

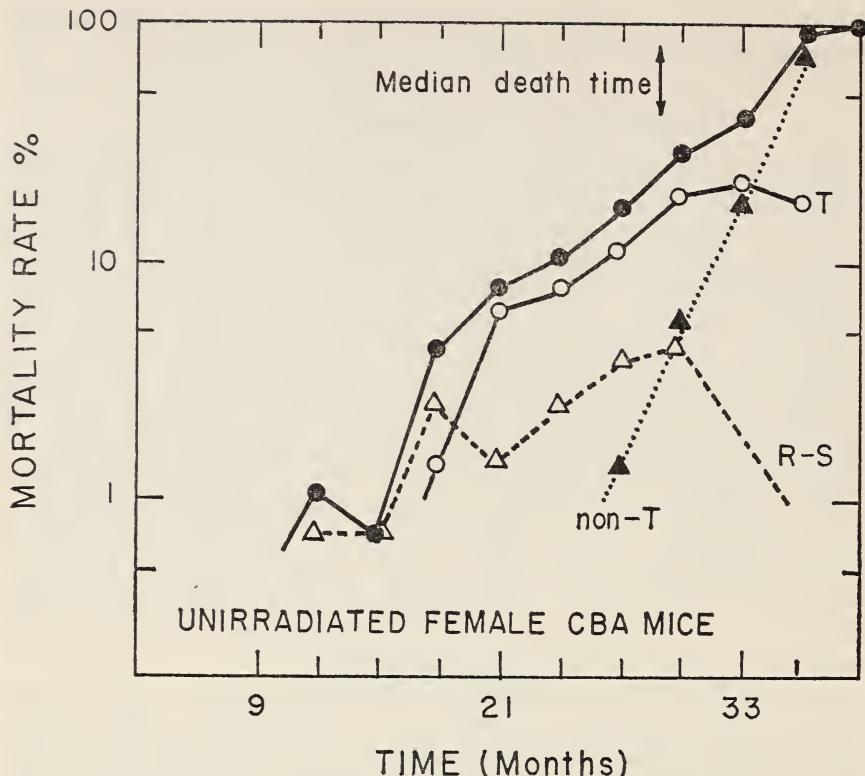
The mortality rates in text-figure 3 do not resemble at all the corresponding human picture. Tumors predominate in the mice, accidents (included in nontumorous causes) are very rare, and so are recognizable infections. Laboratory mice housed in good conditions live a much more sheltered life than the most sheltered and self-regarding human being who might well die from sheer boredom in similar conditions.

In female CBA/H mice the leukemia rate increases little with increasing age, but the over-all tumor mortality rate much more so. The progressive increase in tumor rate with increasing age (text-fig. 3) is in line with current ideas that time itself increases the risk of cancer development because of "spontaneous" changes in cells. The most striking phenomenon in old age is the rapid increase in mortality from nontumorous causes associated with a suggestive decrease in tumor mortality rate, certainly a decrease in its previous steady rate of increase. One possible deduction is that the soma of the elderly mouse is an unfruitful environment for tumor growth. Alternatively, animals with tumors may die selectively early. The most common killing tumor type is the hepatoma, and it has been suggested before that hepatomas may actually degenerate with increasing age (5).

Tumor Mortality and Incidence Rates After a 4-Week Period of Fractionated Gamma Radiation

To get groups of mice numerically large enough for meaningful calculation of age-specific rates in middle and late life, all the mice given 1000 r at 0.3 r per minute have been grouped and similarly all the mice given 2000 r at 0.3 r per minute.

Leukemia.—Grouping the data naturally obscures the effect of fractionation and its interaction with total dose which were mentioned earlier. The data presented in text-figure 4 show that the maximum leukemia

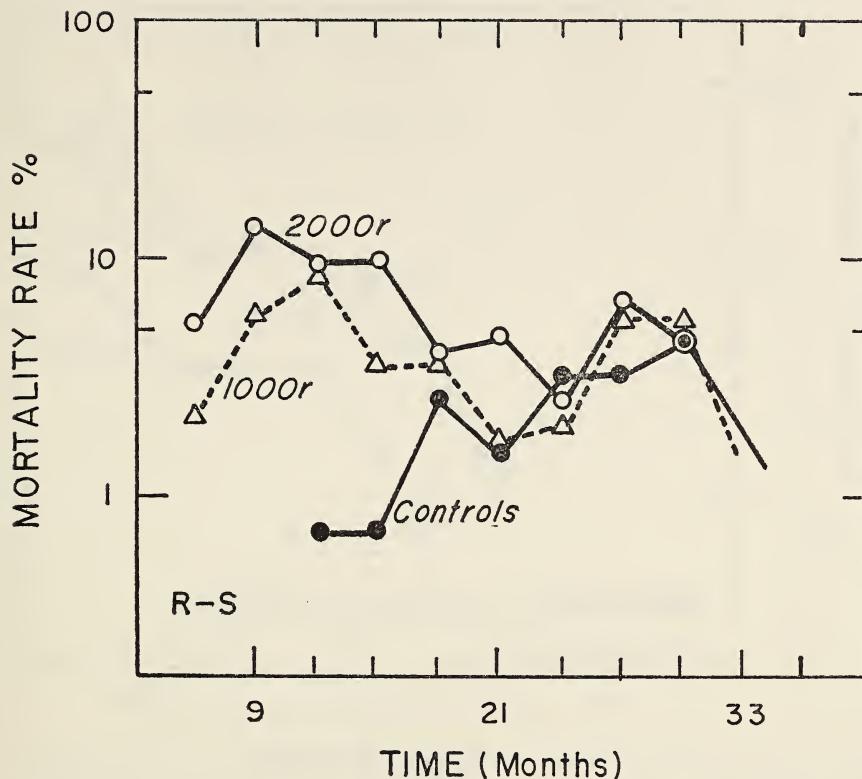


TEXT-FIGURE 3.—Age-specific mortality rates for various causes in unirradiated female CBA mice. In this and subsequent text-figures mortality rates (%) are the ratio of the number of mice dying within successive nonoverlapping 3-month periods divided by the number of mice alive at the beginning of each period. When there was no death or only 1 mouse died in the period the point is not plotted but the line is drawn to give an indication of the trend. Zero time is the start of the experiment when the mice were 60 days old. All causes ●—●; malignant disease of reticular tissue Δ—Δ; all other tumors (benign and malignant) ○—○; causes other than tumors (including unidentified causes) ▲ . . . ▲.

mortality rate is reached relatively very early, perhaps later after 1000 r than after 2000 r, and that thereafter there is a steady decline in the radiation-induced rate. By 24 months the rate is not significantly greater than in unirradiated controls. The effect of the radiation exposure is thus exhausted by an age at which spontaneous leukemia can still present itself.

The general pattern is rather different from that found by other workers under other situations, *e.g.*, in LAF₁ or RF mice after single doses of X or γ radiation, but the pattern of leukemia development with time in unirradiated controls is also quite different in CBA mice. Further analysis according to type of "leukemia" is clearly needed.

Other malignant tumors.—This category of cause of death includes a wide variety of lesions with a distribution quite different from the cor-

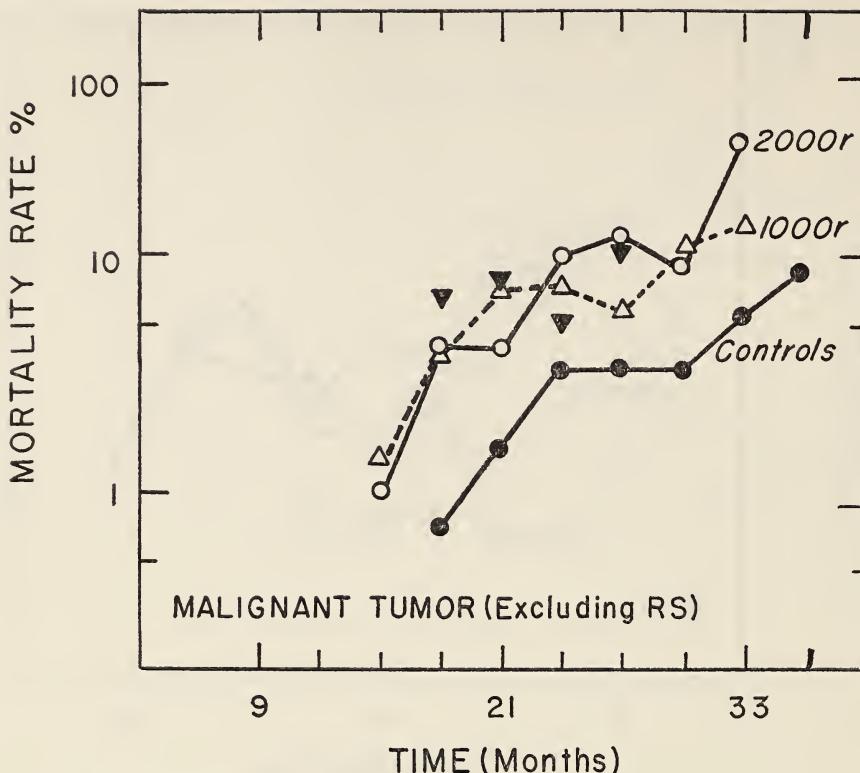


TEXT-FIGURE 4.—Age-specific mortality rates from leukemia in female CBA mice unirradiated or given a 4-week exposure to $\text{Co}^{60}-\gamma$ rays. Data for animals receiving 1000 r Δ — Δ and 2000 r \circ — \circ are shown separately. Control data \bullet — \bullet from text-figure 3.

responding human category. Inspection of the data on malignant tumors in the variously irradiated groups did not reveal any clear-cut fractionation or dose-rate effect comparable to that found for leukemia, and it seemed legitimate therefore to pool the data as already described. However, it may not be safe to conclude that there were no fractionation effects, since the high leukemia incidence between 6 and 15 months in many of the groups left rather small numbers of mice at risk of tumor development.

Text-figure 5 shows clearly that 2000 r had little if any more effect than 1000 r. There seems to be some sort of saturation effect for malignant disease of nonreticular tissue as well as of reticular tissue. Nothing can be said as yet about the effect of 500 r or about the shape of the dose-response relation. The difference in instantaneous dose rate between 0.3 and 1 r per minute seemed to make no difference in the mortality rate from malignant tumors.

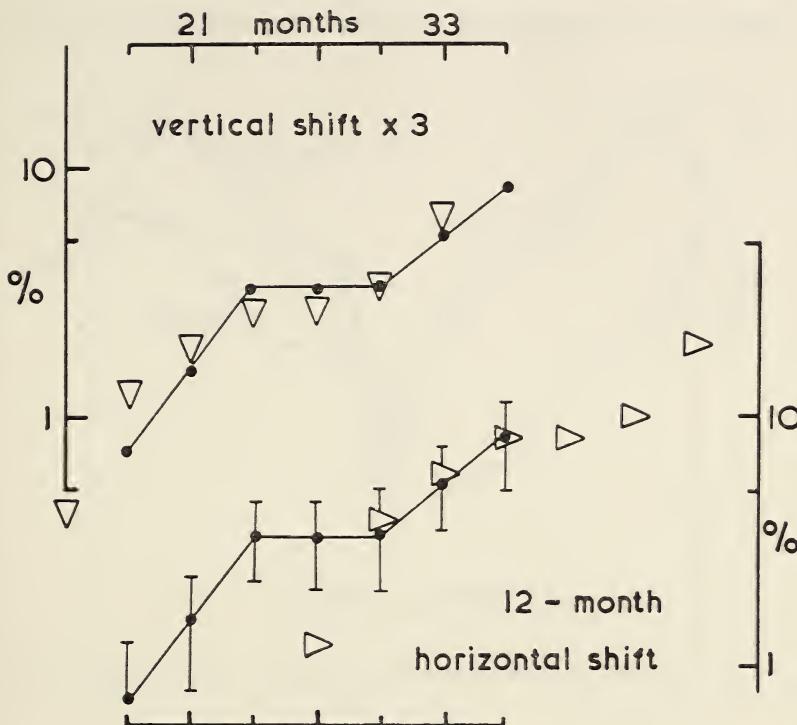
The curve of age-specific mortality rate from malignant tumors against time (age) was very similar in shape in the unirradiated and the irradiated



TEXT-FIGURE 5.—Age-specific mortality rates for malignant tumors (excluding leukemias) in female CBA mice unirradiated or given a 4-week exposure to $\text{Co}^{60}\gamma$ rays. Malignant tumor defined in text. Controls ●—●; 2000 r ○—○; 1000 r at 0.3 r per minute Δ---Δ; 1000 r at 1 r per minute ▼.

mice, even to the tendency to plateau in the middle (text-fig. 5). It may be thought that the effect of radiation was to displace the curve to an earlier time (age); it has often been considered that such a displacement in time shows that radiation exposure causes some kind of "aging." However, when the test is made (text-fig. 6) this sort of displacement gives a worse fit than that given by a vertical displacement, though it should be emphasized that the data are not yet good enough to discriminate with certainty between the two possibilities.

Since the ordinate in text-figures 5 and 6 is on a logarithmic scale, it seems fair to conclude from the approximately equal vertical displacement at each time period that the radiation exposure has had the effect of multiplying the natural spontaneous incidence of cancer by about 3. Further, this consequence of a relatively short radiation exposure in early adult life seems to be maintained very nearly to, if not right up to the end of, the natural lifespan in contrast to the effect of the radiation exposure on "leukemia," which seems to wear off during middle age (text-fig. 4). The tentative suggestion that a time-limited exposure to radiation

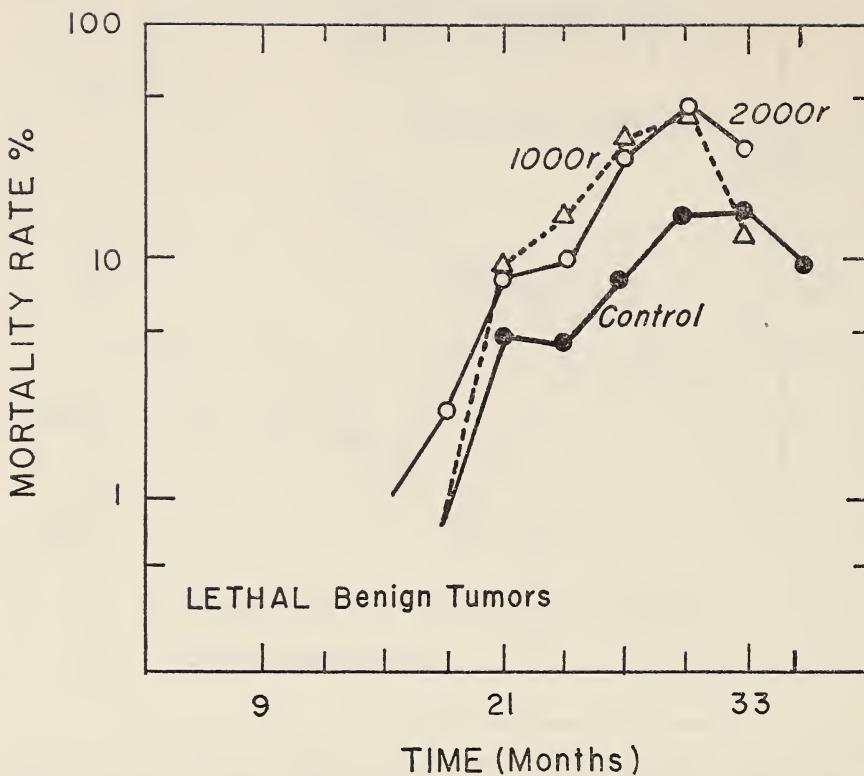


TEXT-FIGURE 6.—Diagrammatic comparison of age-specific mortality rates for malignant tumors in irradiated and unirradiated female CBA mice. Control data from text-figure 5 with standard error indicated by bar. All the data in text-figure 5 for irradiated mice were combined and plotted as open triangles.

might be expected to multiply the spontaneous cancer rate has already been made (3) and will be discussed later.

Other types of tumors.—Hepatomas were by far the most common lethal benign tumor. The mortality rate from all lethal benign tumors together was several times that from nonreticular malignant tumors both in unirradiated controls and in the two irradiated pools. There was a similar but smaller vertical displacement of the irradiated as compared with the unirradiated (text-fig. 7). Again the radiation exposure had a multiplicative effect.

When mortality rates were calculated each individual animal scored 1, however many tumors it might carry. On the other hand, when tumor incidence rates were calculated, each tumor type was recorded separately, irrespective of how many other kinds of tumor an individual mouse might carry. However, no attention was paid to how many different examples of one type of tumor there were in one mouse, *e.g.*, if there were one or several pulmonary adenomas or hepatomas only one was counted. It should be emphasized that there is a profound logical difference between a mortality rate calculated for causes of death and an incidence rate for nonlethal tumors. The latter depends on the "accident" of the animal's



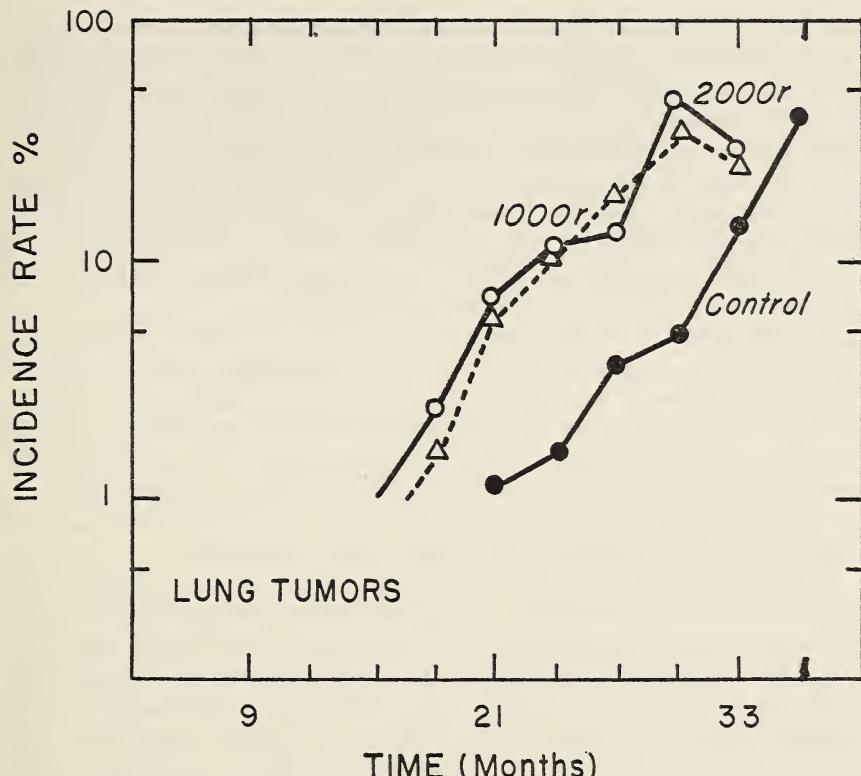
TEXT-FIGURE 7.—Age-specific mortality rates for lethal benign tumors in female CBA mice unirradiated or given a 4-week exposure to $\text{Co}^{60}\text{-}\gamma$ rays. Symbols as in text-figure 4.

dying within the time period under consideration and is meaningful only if, among other things, the induction of the nonlethal tumors is totally independent of the cause of death.

All pulmonary adenomas were confirmed histologically to avoid mistakes of confusion with other lesions, such as metastases of other tumors, and the incidence rates are shown in text-figure 8. Radiation had broadly the same multiplicative effect as before. Except for the interesting decrease in rate in the irradiated groups in advanced old age, there was a fivefold increase in tumor incidence at all times (ages).

Hepatomas and adenomas of the harderian gland were macroscopic, not microscopic diagnoses. Both types of tumor showed the same sort of vertical displacement of incidence rate, by twofold for hepatomas and eightfold for harderian adenomas. The hepatoma data need careful reanalysis since there seems to have been not only a "cage" effect but also a secular change in incidence in controls.

Apart from ovarian tumors, other types of nonmalignant tumors occurred too rarely for mortality rates to be worth calculating. Over-all incidences, calculated as in table 2, showed that adrenal and renal ade-



TEXT-FIGURE 8.—Age-specific incidence rates for pulmonary tumors in female CBA mice unirradiated or given a 4-week exposure to Co^{60} - γ rays. Symbols as in text-figure 4.

mas were increased in the irradiated mice some 15- to 20-fold over the control level.

No type of tumor was significantly decreased in incidence in the irradiated groups.

DISCUSSION

The observations on CBA mice discussed here may be considered to fit in with the hallowed, if no longer generally accepted, tradition that leukemia and cancer are in some sense fundamentally different. In the experiments described they share at least one property: The incidence of induced disease can reach a saturation level which is not exceeded when the carcinogenic exposure is further increased. This of itself shows that the appearance of overt malignant disease depends on more than just a single cellular "event."

If the basis of neoplasia were an infective agent (virus), it might be imagined in general terms that a saturation level of response would be something to be expected for such reasons as that only a limited number of individuals harbored the virus or that continued radiation exposure would

lead to a steady state in which cell numbers and immunity levels were not further impaired by further exposure. However, if it is true, as appears to be the case, that radiation given in the right way (right number of fractions, instantaneous dose rate, total dose) can produce virtually 100 percent leukemia in any strain of mouse (9) then infection with the hypothetical agent (virus) must be nearly, if not quite, universal and the more interesting questions become those about the trigger mechanisms, which determine the activity of the hypothetical agent. It has not yet been possible experimentally to produce 100 percent incidence of cancer as it has to produce 100 percent incidence of leukemia.

The quantitative importance of host factors in murine leukemia has been established for a long time. If the carcinogenic agent itself can modify the host so as to delay, decrease, or delay and decrease the appearance of overt disease, then experimentalists must be cautious in drawing conclusions not only from over-all incidence rates but also from age-specific tumor rates. Whether host factors affect tumor production, as well as leukemia, seems an unexplored field. There must be some explanation for the "saturation" phenomena just discussed.

Penetrating X and γ radiation are unusual experimental tools in carcinogenesis because their time of action can be so precisely delimited; this makes them particularly appropriate as tools to investigate the fairly recent ideas of Armitage and Doll (1). They showed that the age distribution of many types of human cancer fitted the hypothesis that two separate intracellular events (? mutations of some kind) were all that was needed as long as the first event gave some selective advantage to the cell over its unchanged peers. Such a two-event hypothesis seems to fit the experimental facts on bone carcinogenesis in mice by radioactive strontium (3, 16), the short lifespan of the mouse and the high tumor incidence fortunately making it possible to ignore the complications in the hypothesis due to the postulate of selective advantage.

Armitage and Doll (1) assumed that there was normally a constant probability of occurrence at all times of each of the postulated events, though nothing needed to be assumed about the relative probability of the two kinds. If radiation can produce the same kinds of event as occur "spontaneously," and if radiation can cause the first kind of event much more easily than the second kind, then the effect of a time-limited exposure to radiation should be to multiply the natural incidence rate of cancer at all subsequent ages. It was suggested (8) that this idea was compatible with the reported incidence of cancer in atom-bomb survivors at Hiroshima (17). Perhaps the experimental evidence presented here on tumors of nonreticular tissue lends further support to the idea. It is naturally heterodox to consider any carcinogen as multiplying rather than adding to a carcinogenic risk, but there are several examples from human experience in which the arithmetic excess of induced tumors increases with an increase in the age of exposure to the carcinogen (18).

If radiation were to cause the second kind of event much more easily than the first, then the effect of a time-limited exposure should be to

produce an early increase in incidence followed by a steady decrease to the normal level for the age under consideration. The carcinogenic effect would become exhausted in time. This is, in fact, the pattern of response of human radiation-induced leukemia in atom-bomb survivors in Japan, in ankylosing spondylitics, and in children exposed to antenatal radiation (19) and is also shown in the murine leukemia data of text-figures 2 and 4. It may be suggested that, although there may be no fundamental difference in the nature of acute leukemia and cancer, there is a marked difference in their epidemiology because of the difference between reticular and other stem cells in the relative probabilities of the first and second events (either spontaneous or radiation-induced) which are needed before cells behave as truly malignant cells. Pursuing the notion still further, is it possible that chronic lymphatic leukemia of man and some, if not all, chronic myeloid leukemias of man are not fully developed malignant diseases but halfway states, so to speak, in which the first event only has occurred and demonstrating in the accumulation of cells in the tissues that the first kind of event does indeed lead to the sort of selective advantage that according to Armitage and Doll (1) must exist? Acute leukemic transformation of such chronic and only mildly disabling conditions would correspond to the occurrence of the second kind of event.

Recent interest in the cytogenetics of leukemia and primary tumors has led to increasing evidence that chromosomal abnormalities are characteristic of developed malignant disease (20-22). This has an immediate appeal to anyone interested in the idea that carcinogenesis is based on more than one separate intracellular event, for the kinds of chromosomal abnormalities observed should be much more easily produced by multiple than by single events. Much more extensive and detailed information is needed on this subject, as also on the different individual types of cancer which can be produced by exposure to radiation, before one can tell whether the possible clues discussed in this paper really do increase our understanding of cancer. It is probably true that we are still in the stage of establishing what chronic radiation does, and that investigation of mechanisms must wait until we know more exactly what needs to be explained.

RESUMEN

Cuando el efecto carcinogénico de un agente exógeno como la radiación se mide por el cambio en la incidencia tumoral de conjunto, comparada con los controles, puede conducir a una impresión equivocada. La mortalidad específica por edad o tasas de incidencia pueden ser más reveladoras y se han usado para trazar las siguientes conclusiones.

En experimentos en que las hembras de ratones CBA fueron expuestas a radiaciones gama fraccionadas de todo el cuerpo por un período de 4 semanas a una dosis total de 1000 ó 2000 r, el efecto leucemiogénico aparece temprano y se agota en 2 años. En el mismo experimento la radiación parece actuar diferentemente en la inducción de otros tipos de tumor, malignos ó benignos. Las tasas de mortalidad específica por edad para estas lesiones parecen estar aumentadas en una proporción constante en todas las

edades, es decir que la radiación tiene una acción multiplicativa. No hubo reducción, ó solamente muy pequeña, en esta acción carcinogénica aún en edad avanzada y por consiguiente no hubo agotamiento con el tiempo. Esta diferencia experimental entre leucemia y otras formas de neoplasia se refleja en la experiencia humana.

Tales resultados pueden ser quizá entendidos en la hipótesis de que la carcinogénesis depende de dos eventos celulares sucesivos, cada uno de los cuales puede ocurrir espontáneamente y cada uno de los cuales puede ser causado por la radiación, con la suposición adicional que las probabilidades relativas de las dos clases de eventos son diferentes para leucemia y para otras clases de tumor.

Cualquier hipótesis adecuada de carcinogénesis debe explicar cómo un aumento en la cantidad de exposición carcinogénica (radiación) puede fallar en conducir a un mayor aumento en la tasa tumoral. Tanto la leucemia como los otros tipos de tumor muestran este tipo de saturación de la respuesta.

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DISCUSSION

Kaplan: I think you said that the cancers and other sarcomas were real tumors in the sense that they were invasive and showed metastases. Apparently your reference to real tumors distinguishes the lymphomas, leukemias, and reticulum cell tumors from real tumors. This suggests that they do not invade and do not metastasize. I would have been deeply shocked if I thought you really meant that. Perhaps you would clarify this. The point on which you asked for a comment had to do with the apparently smaller effect of 2000 r versus 1000 r in one of those elaborate experiments, and I think you supplied the answer yourself. First of all, a little arithmetic convinced me that the animals getting 2000 r in 4 weeks at 1 r per minute were under the beam for about 3 hours or so per day; 2 to 3 hours per day might possibly interfere with their nutrition during the experiment. Secondly, you said that the mortality was significantly higher in the 2000 r group and that their weights relative to the 1000 r group were severely reduced. Now we know, from the work of Tannenbaum and others on the relation of caloric restriction and underfeeding to lymphoid tissue growth and thymus growth in particular, that one of the most severe involutional stresses the thymus can be subjected to is starvation or malnutrition. We know that any kind of involutional influence having the effect of shrinking the thymus will reduce lymphoid tumor incidence. The effect is dependent on how long the involutional stimulus is maintained. If it is maintained briefly one sees only a delay in the development of thymic lymphomas and the incidence climbs again afterward. If it is maintained, as we have done with testosterone, for 10 weeks or longer, then the incidence remains very low.

Mole: I am sorry if my slide was misleading. I separated malignant disease of reticular tissue from other kinds of tumor for reasons which I expect are clear, and called them all "hematosarcomas" (cf Mathé, *Bull WHO* 26: 585, 1962). Thus "other" in the category of "cancers and other sarcomas" merely means nonreticular. I agree that all hematosarcomas metastasize and are invasive.

I agree that involutional influences acting on the thymus may indeed modify tumor incidence. However, the thymus gland of animals given 2000 r (at an instantaneous dose rate ~ 1 r/min) may return to a normal size within a few weeks without leukemia developing within 400 days. In fact, over-all mortality at this time was much less than in mice given 1000 r simply because leukemia did not occur. One interesting point is that some leukemia eventually developed in the 2000 r animals but after 400

days, the time at which Kaplan normally gets rid of his surviving animals. Surely even the standard leukemogenic course of irradiation of C57BL weanlings, used by Kaplin and later by others for the important work on leukemogenesis in unirradiated thymic grafts, etc., is an exposure which leads to a permanent loss in weight.

Upton: Your study emphasizes the enormous complexity of the experimental system in animals when one looks at variations in total dose and dose rate as they affect survival and the probability of different kinds of physiopathological changes, including neoplasia. These kinds of experiments are extremely difficult to design and interpret. Their interpretation, in terms of basic mechanisms, has been further complicated by recent work in genetics from several laboratories, notably that of W. L. Russell. As was mentioned earlier, an effect heretofore considered to be a single-hit is now, at least insofar as its expression is concerned, clearly not this way; *i.e.*, properly timed fractionated doses of radiation have been shown by Russell (Proc Nat Acad Sci USA, 48: 1724, 1962) to yield a greater number of mutations than the same amount of radiation given in a single exposure. But when the dose rate is decreased sufficiently, the mutagenic effectiveness of the radiation is reduced. Here one is contending with, presumably, repair (call it repair of some sort) at the subcellular level at low dose rates. But at higher dose rates there may be some sort of staging of cells, if you will, or selection, going on that heightens the responsiveness of the system in question, and, as one increases the total dose and dose rate above certain levels, cytotoxic effects interfere with the expression of the other processes mentioned. It seems to me that all of these factors have to be considered in the design and interpretation of an experiment, which thus becomes extremely difficult. Extrapolation from single dose studies to chronic dose studies, or from experiments at high dose rates, to exposure conditions ordinarily operating in the human environment pose enormous problems indeed.

Tannenbaum: At a symposium concerned with carcinogenesis, held in Rehovoth, Israel, about 4 years ago, I reported investigations demonstrating the multipotential carcinogenicity of urethan. A colleague chided me for using a messy chemical for such investigations, and asked why nice clean radiation was not utilized. Obviously, these remarks were not entirely serious. However, the presentation by Dr. Mole and others before him indicates that whole-body radiation can also be messy. Both chemicals and radiation produce numerous effects—some recognized, others not—and often attention is directed only to the observation of the formation of tumors. In relatively short-term investigations, in which a potent carcinogen is used in high dosage, a large incidence of a specific type of neoplasm may result. These may cause death of the animals which, when coupled with early termination of the study, preclude the possible appearance of other types of tumors that occur at a later time in the lifespan. By proper, sometimes fortuitous, choice of the experimental animal, dosage and periodicity of agent (chemical carcinogen or radiation), and by extending the duration of the experiment, one should be able to effectuate the production of tumors late in the lifespan so that the appearance of one type does not interfere too much with the appearance of other types. Under such conditions one can observe and evaluate the neoplastic potentiality of the agent in influencing the formation of many, *individually recognizable* types of neoplasms.

Observations on Possible Mechanisms of Leukemia Induction in Man Following Radiation Exposure¹

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SUMMARY

Studies of the mortality from leukemia in heavily irradiated human populations are discussed in the light of possible mechanisms of radiation leukemogenesis. Account is also taken of newer knowledge from human cytogenetics, particularly in regard to the finding of a specific chromosome abnormality in most cases of chronic myeloid leukemia and the remarkable persistence for many years of chromosome abnormalities produced by *in vivo* radiation exposure in man and as seen in blood cultures. A more recent

development is the study of the cytogenetics of human cells transformed *in vitro* by exposure to the SV40 and herpes simplex viruses, and also the study of the *in vivo* production of certain forms of chromosome damage in man following infection with measles and an attenuated form of the yellow fever virus. It is suggested that possibly in man the action of radiation in the production of leukemia may be to facilitate the transformation of cells by a leukemogenic virus.—Nat Cancer Inst Monogr 14: 291-300, 1964.

FOR A number of years my colleagues and I have studied the long-term effects of radiation damage in man, particularly the induction of leukemia. This study led us to the field of human cytogenetics, and for the last 5 years we have been examining a wide range of cytogenetic abnormalities in man. In this paper I will present some observations on possible mechanisms of tumor induction following radiation exposure, and will draw together some of the varied leads that are arising from such seemingly diverse topics as the epidemiology and cytogenetic effects of radiation injury, the cytogenetic structure of leukemic cells, and the newer data of the effects of viral transformation on chromosome structure.

EPIDEMIOLOGICAL TECHNIQUES AND THE MECHANISMS OF TUMOR INDUCTION

Often there is some merit in the historical approach. Since the early 1950's many attempts have been made to relate, in an exposed human

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population, the incidence of leukemia to the radiation dose, in the hope that the nature of the dose-response relationship would shed light on the mechanism of induction of leukemia. In 1954 my colleague, Richard Doll, and I were asked to provide the Medical Research Council with some indication of the possible dose-response relationship for the induction of leukemia mortality in man by radiation. We had at our disposal some 13,000 case records of subjects who had been given partial-body exposures, in the form of spinal axis irradiation, for a rheumatic disease known as ankylosing spondylitis.

Finkel and Mole have stressed that the yield of tumors are influenced by such variables as total dose, intensity of irradiation, and the system of fractionation of exposure (see Finkel and Mole, this Symposium). Our task was seriously complicated because our subjects were treated at 82 British radiotherapy centers, which meant that there were considerable variations between centers in the proportion of the trunk irradiated, the total dose, the system of fractionation, and the dose rate or tube intensity with which each fraction was given. In addition an important piece of information was lacking: We did not know whether, after a single radiation exposure, an individual was equally liable to the development of leukemia in each postexposure year for the rest of his life, or at any rate for a very long time, whether this risk was only incurred over a limited duration of time, say a decade, or whether it changed in some fashion or other from year to year. The existing data, derived from the study of the Japanese survivors, suggested that after rising for the first year or two the number of cases thereafter remained relatively constant.

We finally postulated that the induction of leukemia was the result of a single irreversible event in an individual cell, endowing it with malignant or potentially malignant properties. According to thinking and experimental experience in those days, this implied a situation analogous to the induction of gene mutations by radiation, and it followed that the rate of accumulation of dose would not affect the proportional relationship between dose and response. This was important to us since many of our subjects had been given more than one course of X-ray treatment. It also followed that the most suitable parameter of dose was the mean dose throughout the target organ, in our case the spinal marrow. Again our postulate allowed us, assuming the spinal marrow represents some fixed fraction of the total body marrow, to calculate from our results the effect of a mean total-body marrow dose of 1 rad.

Our findings, at least for the lower ranges of dose that we studied, were compatible with a linear relationship, and on this basis we calculated that a mean whole-body marrow dose of 1 rad would produce between 1 and 2 cases of leukemia per million men per year (1). I do not wish to go into the various criticisms and objections to our approach to this problem, but I do want to raise a criticism of my own which stems from our more recent knowledge of the cytogenetic structure of leukemic cells.

Experience is now teaching us that we cannot treat all cases of leukemia, occurring in an exposed population, strictly on the basis that they are due

to a single event produced by radiation in the genome of the cell. If radiation-induced chromosome damage is solely responsible for the development of chronic myeloid leukemia, then our present cytogenetic knowledge about this disease indicates that we cannot assume it to be induced by a single event; it must be at least two events. I am referring, of course, to the possible mechanism of formation of the Philadelphia chromosome, which will be discussed later.

Radiation exposure certainly increases the risk of development of chronic myeloid leukemia, unlike that of chronic lymphatic leukemia; in fact, among the survivors in Hiroshima under 1500 meters from the hypocenter, there were almost as many cases of chronic myeloid leukemia as acute leukemia. Furthermore, the mean latent period for chronic myeloid leukemia appears to be about 1 year less than that for the acute leukemias. I think it follows that any future attempt to evaluate the dose-response relationship for leukemia will have to be made separately for the chronic myeloid form of the disease.

Perhaps it may be foolhardy, but one wonders if we have not temporarily reached the limits of the epidemiological approach from the standpoint of deducing possible mechanisms of induction. I believe that the epidemiological approach may well prove of value, but only after we have learned more about the sequence of events at the cellular and subcellular level.

THE PROBLEM OF SPECIFIC CHROMOSOME ABNORMALITY IN CHRONIC MYELOID LEUKEMIA

I regard the discovery of a specific chromosome abnormality in chronic myeloid leukemia as one of the most exciting developments in human cytogenetics during the last 2 or 3 years (2-7). Chronic myeloid leukemia appears to be a leukemia peculiar to man, having no clear counterpart in the animal leukemias. It is also the only known naturally occurring human or animal tumor with a recognizable and specific genetic marker in its cells. The presence of this marker, the Philadelphia or Ph^1 chromosome, permits the study of the evolution of a tumor, at least from the time it presents clinically to the time that it causes death.

The direct marrow technique (8), a technique for the preparation of suitable metaphase cells without a preliminary culture period, has revealed the following facts:

(i) Most cases, possibly not less than 95 percent, show the Ph^1 chromosome. Those that do not show the Ph^1 chromosome remain a mystery, but it may be that some different method of induction is involved.

(ii) The study of other tissues as well as marrow and blood indicates that the abnormal chromosome arises *de novo* in the hematopoietic tissues and is not a constitutional abnormality.

(iii) The Ph^1 positive (Ph^1+ ve) cases, based on the findings when first seen in the untreated chronic stage, are further divisible in 3 sub-

groups: (a) those showing no abnormality, apart from the Ph^1 chromosome (? about 65 %); (b) those showing an additional abnormality, either another structurally abnormal chromosome, or another Ph^1+ ve cell line distinguished by chromosome number, or a combination of both (? about 30%); (c) a rare group of males in whom the marrow is wholly or partly constituted of XO Ph^1+ ve cells. Where another line is present this consists of Ph^1+ ve diploid cells.

(iv) In Ph^1+ ve cases virtually every diploid or near diploid metaphase cell in the bone marrow is Ph^1+ ve. The polyploid cells are also Ph^1+ ve, and the frequency of these latter cells directly correlates with the blood platelet level so that these can be accepted as megakaryocytes. We suggested that erythrocyte precursors would be likely to be Ph^1+ ve, and since then Trujillo and Ohno (9) and Whang *et al.* (10) have produced evidence for this. Thus, everything points to the 3 main lines of marrow cells being Ph^1+ ve, and suggests an origin from a common ancestral cell.

Although this may appear somewhat irrelevant to the problem of radiation and cancer induction it is not, for we must consider the role of the Ph^1 chromosome, if any, in the induction of chronic myeloid leukemia. In this regard we have to remember that cytogenetically there appears to be no difference between cases of chronic myeloid leukemia not thought to be radiation-induced and those considered to have a high probability of being so induced, at any rate from the study of a limited number of cases.

A simple argument is that radiation induces the disease by causing a deletion of material from the long arm of a small acrocentric autosome, thought to be a member of pair 21, following the production of two breaks. This deletion in some way puts the cell and its progeny at an advantage to normal diploid cells in the marrow, so that there is a steady and eventually complete replacement of the normal by the abnormal, and this affects the 3 major cell lines in the marrow. That the normal cells may only be in a dormant state is suggested by the finding that normal diploid cells reappear in the marrow under the circumstances in which marrow failure develops during or after treatment with X rays or chemotherapy. Other explanations for this, however, are possible. The natural termination of chronic myeloid leukemia is one of transition into acute leukemia and often this change is accompanied by the appearance of more bizarre cell lines in the marrow, all Ph^1+ ve. Thus another feature of Ph^1+ ve diploid cells may be an inherent instability, so that errors at division are likely, and in this way there is an accumulation in the tumor of further abnormal lines, in some way linked with the clinical evolution of the disease into the acute state.

THE PROBLEM OF VIRAL TRANSFORMATION

Assuming the basic change to be the origin of the Ph^1 chromosome, then if radiation is the only agent which can effect this change we would

have to postulate that all Ph^1+ ve cases of the disease were radiation-induced. This, of course, cannot be disproved, but on the whole it seems unlikely. The problem can be circumvented if two or more agents can produce the same form of abnormal chromosome, and there is some evidence that this may be true. Thus Hampar and Ellison (11) have recently reported on the location of chromosome breaks in hamster cells following infection with the herpes simplex virus. They state that the breaks are located at loci morphologically indistinguishable from those at which breaks occur after exposure of the cells to 5-bromodeoxyuridine, hydroxyamine, and X rays. Moorhead and Saksela (12) have considered much the same problem in a study of chromosome aberrations in SV40-transformed human fibroblasts. They found evidence for a nonrandom production of aberrations, with possibly preferential implication of specific chromosome regions by chromatid and chromosome breaks at the sites which normally bear secondary constrictions. There was also evidence for the particular involvement of one or other pair of the small acrocentric autosomes, with the finding of monosomy for one of these pairs. Shein and Enders (13) found evidence implicating these same autosomes in SV40-transformed human kidney cell cultures.

However these findings are from *in vitro* systems, and the question arises of what may be found *in vivo* following viral transformation. Considerable excitement has been caused by the report of Nichols and his colleagues (14) of a marked increase in chromatid and chromosome breaks and gaps in cells from blood cultures taken from children on or about the 5th day after the appearance of the rash in measles. In my Unit, Harnden (15) has tried to repeat these observations—so far without success—but he has been able to demonstrate what must be a similar effect in a proportion of individuals inoculated with an attenuated yellow fever virus. In a number of these subjects, about the 8th or 9th day after inoculation, there was a very high proportion, in one instance up to 80 percent, of cells showing chromatid and chromosome breaks. These changes seemed to persist for about 24 to 48 hours and then disappear. The effect was clearly a significant one as we had never seen anything like it in over 2,000 blood cultures, but it seemed very variable, and so far all attempts to understand why one person showed it, while another did not, have failed. However, the experience with measles and with yellow fever virus suggests that this direct approach to the study of *in vivo* effects in man of viral infections may be rewarding.

If eventually it is substantiated that a number of viruses produce this effect, particularly viruses as common as those of measles or rubella, then the question arises whether every now and again an end result of such infections is the production of genetic change in a stem cell, which may be visible sometimes as an abnormal chromosome or chromosomes and ultimately lead to tumor production. One might imagine that if this can occur, and, bearing in mind the evidence for selective chromosome damage after viral transformation, then study of tumors produced in this way might show them to have only a limited number of genotypes.

In this regard, another approach is being made to this problem by my colleagues, Miss Jacobs and Miss Tough, through the study of subjects with Burkitt's lymphoma. The climatic limitations on the distribution of this disease in Africa suggest that it is a vector-borne disease and, therefore, possibly of viral origin. The study of direct preparations from the tumors shows evidence for chromosome changes though not in all tumors, and it is possible that where changes are present they may be of limited types. However, too few cases have been studied to be certain of this.

It appears, therefore, that: 1) A number of different agents may produce the same type of chromosome abnormality as X rays; 2) there may either be sites of predilection on the chromosomes for such damage, or, alternatively, if the damage is random certain forms are more compatible with survival than others; and 3) the whole field of viral transformation and cytogenetic damage may be rewarding in the consideration of tumor etiology.

CHROMOSOME DAMAGE FOLLOWING *IN VIVO* EXPOSURE AND LEUKEMIA INDUCTION

Finally, I would like to return to the problem of leukemia induction in a heavily irradiated human population. One of the mysteries of this process is its apparent randomness, there being no obvious reason why one individual rather than another develops the disease under similar conditions of exposure. Recent advances in cytogenetic techniques have made possible the demonstration of chromosome damage produced in lymphocytes following *in vivo* radiation exposure. At Oak Ridge, Bender and Gooch (16, 17) have shown the presence and persistence of such damage in the survivors of the Y-12 criticality accident. They demonstrated the striking fact that cells carrying essentially unstable chromosome abnormalities, such as dicentrics and fragments, were still present as long as 42 months after exposure.

In my Unit, Buckton and her colleagues (18) have made a similar study on patients treated with X rays for ankylosing spondylitis. By studying subjects who had received only one course of treatment to the spinal axis, and this at any time from 1962 to as far back as 1943, we were able to build up a picture of how the different forms of chromosome abnormality changed with time. It was found that abnormalities could persist for at least 20 years, and that two major subdivisions of abnormalities differed from one another in their behavior. Cells carrying abnormalities such as fragments, dicentrics, tricentrics, and ring chromosomes, regarded as unstable because such abnormalities would be expected to disappear after a few divisions, rose to a peak shortly after exposure and thereafter fell away exponentially. They did not, however, reach near control levels until between 7 and 8 years after exposure. Cells carrying stable chromosome rearrangements persisted for over the 20-

year period at much the same level as they had reached on the cessation of irradiation. This latter finding, however, may be rather misleading as in the earlier years higher total doses of X rays had been given by comparison with the later years, so that a tendency for cells with stable abnormalities to decrease in number may have been offset by these higher doses.

We presume that qualitatively the same types of abnormality are produced in other tissues, particularly the bone marrow, so the question arises of the possible significance of these varied abnormalities to the induction of leukemia. We did not find evidence for clone formation, using the term in the context of a cell line, at a particular advantage to other lines, nor did we find evidence for the survival of unstable cells once the unstable type of abnormality had been lost. But a total of only 58 subjects was studied, so that we cannot exclude the possibility that every now and again radiation alone may be responsible for the initiation of a clone of cells which ultimately produces frank malignancy. However, other possibilities also suggest themselves, if we are going to consider viral transformation as an essential step in the genesis of at least some tumors. Radiation may act by producing a cell line of such an altered genotype that the cells are sensitive to transformation by a pre-existing and apparently harmless virus. Another possibility arises from the observations of Law and Dawe (19), who showed that total-body exposure in combination with the polyoma agent produced salivary gland tumors in adult mice whereas neither agent alone was effective. Such an effect presumably could depend on the production of cells of an altered genotype, but it could also depend on the modifications by radiation of immune responses. This latter possibility is again one for consideration in relation to man. Thirdly, radiation may act directly on a pre-existing virus so that it became capable of transforming either normal cells or cell, the genotype of which had been altered by radiation.

If any of these ideas have any merit at all, they may act as a link between what, up until now, have tended to be contrasting and even opposing opinions about leukemogenesis. That is, between those who have propounded a viral etiology and those who have taken their stand on radiation-induced leukemia being solely the result of radiation-induced genetic change.

RESUMEN

Los estudios de mortalidad por leucemia en poblaciones humanas intensamente irradiadas se discuten a la luz de los posibles mecanismos de la leucemogénesis por radiación. También se ha tomado en consideración los más recientes conocimientos de la citogenética humana, en particular sobre el hallazgo de una anomalía específica cromosomal en la gran mayoría de casos de leucemia crónica mieloide y la notable persistencia por muchos años de anomalías cromosómicas producidas por la exposición *in vivo* a radiaciones en el hombre y como las observadas en cultivos de sangre. Un desarrollo más reciente es el estudio de la citogenética de células humanas

transformadas *in vitro* mediante la exposición al SV40 y al virus de herpes simple, y también el estudio de la producción *in vivo* de algunas formas de daño cromosomal en el hombre consecutivas a la infección con sarampión y con una forma atenuada del virus de la fiebre amarilla. Se sugiere que en el hombre la acción de la radiación en la producción de leucemia posiblemente sería facilitar la transformación de las células por un virus leucemiogénico.

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DISCUSSION

Mole: Is there any reason to think there is a direct association between myeloid leukemia and the Philadelphia chromosome?

Upton: The Philadelphia chromosome, with its deletion, merely represents loss of genetic information. One would expect to duplicate its effects by mutation on the chromosome in question, without having that chromosome fragment missing. So if the Philadelphia chromosome were an etiological mechanism, this might explain its absence in some individuals. Furthermore, if there is a common pluripotent stem cell in the bone marrow, then we would expect cells other than myeloid cells to show the Philadelphia chromosome.

Mazia:* I wonder whether it has been proved that the shortness of the chromosome arm really represents a deletion. Could it not be the result of a higher degree of condensation of that arm?

Court Brown: I agree that one cannot be absolutely certain that there is not a modification leading to a shortening of the long arm of an autosome 21. However, other studies have shown that certain abnormalities, which may be local modifications of heterochromatin, appear to be restricted to autosomes with a secondary constriction and probably to the sites of these constrictions or immediately adjacent to them. The sites involved are the short arms of the long acrocentrics and the short acrocentrics and the long arm of autosome 16. It is not recognized that there is a secondary constriction on the long arm of an autosome 21 and it seems likely, therefore, that the formation of the Philadelphia chromosome is due to the deletion of material.

Kaplan: May I ask whether there has been a very careful analysis on a case for case basis of those cases which fail to show the Philadelphia chromosome and the alkaline phosphatase reaction? Is there a perfect correlation between the alkaline phosphatase reaction and the presence of the Philadelphia chromosome?

Court Brown: A study of the polymorphs of patients not showing the Philadelphia chromosome so far has shown these to be deficient in alkaline phosphatase in the same way as polymorphs containing the Philadelphia chromosome. There is no direct correlation between the amount of autosome 21 material and alkaline phosphatase activity. Suggestions have been made that this is so from study of the activities in polymorphs of normals and mongols as well as in $\text{Ph}^1 + \text{ve}$ cases of chronic myeloid leukemia. However, the suggested correlation breaks down in respect to the $\text{Ph}^1 - \text{va}$ cases, already mentioned, and $\text{Ph}^1 + \text{ve}$ cases of chronic myeloid leukemia which have transformed in cases of acute leukemia. In the latter, the cells after transformation may show grossly excessive alkaline phosphatase activity.

Lamerton: Is it a reasonable assumption that the change occurs in one stem cell and that this then repopulates the whole bone marrow? How long is the process likely to take, and is the necessary degree of selective advantage feasible? Perhaps another possibility is the presence of some sort of agent which produces the specific stem cell abnormality in all the cells.

Court Brown: Our only indication of the time required to repopulate the whole bone marrow by the progeny of a modified stem cell is derived from a study of the latent period in radiation-induced cases of the disease. This can only be a very approximate indication and is based on the assumption that the initial change occurs at

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the time of irradiation. Also it is necessary to bear in mind that complete repopulation of the marrow may occur long before the clinical manifestation of leukemia. However, with these limitations in mind, the latent period on the average lies between 4 and 8 years as measured from exposure to the time the patient first seeks advice.

Upton: The nearly 20-fold increase in susceptibility of mongoloid individuals to leukemia, which is associated with an extra small autosome (possibly 21), suggests further that this chromosome may be involved in the regulation of leukocyte balance. Have you seen in your material or do you know of reported cases of trisomy for this small chromosome in the hematopoietic system in association with leukemia without stigmata of mongolism? Can one get, late in life, trisomy for this chromosome in the marrow, and will that contribute to leukemia? Is there any evidence for this?

Court Brown: We have not to my knowledge seen trisomy of autosome 21 in cases of leukemia other than in mongolism, but our experience of leukemia, apart from chronic myeloid leukemia, is limited.

Luria: After hearing this discussion one might make a suggestion. What may be wrong in these types of leukemia may be a disarrangement of some kind of control system that maintains the integrity of the chromosome complement in the cells. Once a transient or persistent aberration of this regulatory mechanism has occurred, different chromosomes may become lost or duplicated. The important point is that the changes relevant for malignancy may be totally unrelated to those recognizable under the microscope.

Geographic Pathology and Cancer Induction at High Altitude

Geographic Pathology¹

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SUMMARY

In summary, I may say that up to the present time cancer research has focused too sharply on the small laboratory animal, to the neglect of the opportunities that exist for the study of cancer in man in all its varied manifestations. Experience teaches that important contributions to medicine and science have emanated from work based upon ideas derived from human disease. It is important to establish and to maintain this point: that studies in human disease, where nature rather than the investigator has established the experimental conditions, constitute research of the highest order. The lack of money, the lack of enterprise, the general disinterest, and the caution and lack of imagination of

top administrators are all in one way or another responsible for this outrageous neglect of the study of man and his cancers, in favor of the vastly easier method of the study of cancer in small laboratory animals. One wonders how this all came about when one reflects that the goal of medical investigations in cancer is to learn more about human cancer, with the primary hope for the detection on a broad scale, the early diagnosis, the cure, and the control of cancer in human beings. In one way or another, this prejudice against studies of human beings with cancer must be dispelled, so that greater research efforts can be channeled into the field of geographic pathology.—Nat Cancer Inst Monogr 14: 303-308, 1964.

MR. CHAIRMAN, members of the Symposium on the Control of Cell Division and the Induction of Cancer. I should like to consider with you today the opportunities that exist through the methods of geographic pathology for investigative studies of cancer. We know for certain the etiologies of less than 2 percent of cancers that we see in clinical practice today. Virtually all of the discoveries of these etiologies came about as a result of environmental exposures of people in industry. Medical history teaches us that our best hope for the control of diseases is based upon a knowledge of its etiologies and the environmental factors that influence them. Geographic pathology offers a means to study these.

Recent years have witnessed a change in the relative importance of various diseases throughout the world. Today, cancer and the chronic

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diseases of unknown etiology are in many countries replacing the diseases of known etiology. The methods of geographic pathology have been employed too little in the study of cancer. Yet, in the past, the wealth of information that these methods have revealed about the communicable and nutritional diseases has enabled physicians to stamp out illness on a massive scale. Thus, students of the *communicable* and *nutritional* diseases have, through long experience, acquired a sophistication in the pursuit of geographic pathology that students of the cancerous diseases may well seek to emulate.

THE SHREWD GUESS

To illustrate: the solution of scurvy, pellagra, beriberi, and yellow fever and the subsequent emergence of much basic knowledge of vitamins, metabolism, nutrition, neural function, connective tissue growth, and virology exemplify geographic pathology in full operation. Over the span of many centuries, repeated observations had confirmed the onset of scurvy in ships' companies on long sea voyages. For example, Camoens states in his epic poem that 1 in 3 members of Vasco da Gama's crews that sailed from Lisbon to the Far East, on July 8, 1497, was fated to fall victim to scurvy and never see Portugal again. Ashore, in port, or at home, the surviving scorbatic sailors recovered, and, while on land, remained free from scurvy. Different hypotheses arose about the cause, prevention, and cure of scurvy. In 1747, James Lind cured scorbatic patients with dietary supplements of citrus fruits. His discovery constitutes an example of the combination of a shrewd guess and a model epidemiological experiment (using human beings in this case). The natural but tardy sequences of Lind's discovery were the experimental production of scurvy in guinea pigs in 1912 by Holst and Frölich, the inadvertent isolation of ascorbic acid in 1927 by Szent-Györgyi, who at first failed to recognize its vitamin characteristics, and the subsequent isolation and the elucidation of the nature of the curative agent by King and Waugh in 1932.

Pellagra is of comparatively modern recognition. Geographically, it was observed in the peasantry in parts of Italy, France, and Lower and Upper Egypt, among the Zulus and Basutos of South Africa, and in peoples of the southern United States, who subsisted chiefly on large quantities of American corn. Joseph Goldberger, in 1925, demonstrated the lack of certain essential substances in the corn diets of patients with pellagra. Within a dozen years, Elvehjem isolated and identified nicotinic acid or its amide as the vitamin involved in the essential dietary substances that prevented or cured pellagra. Pets that subsisted on the inadequate diets of pellagrous families exhibited an analogous deficiency disease, and this pointed up the etiology of black tongue in dogs.

A similar set of circumstances led to the discovery of thiamine. This

followed upon the observations of the Dutch investigator, Christian Eijkman. Working in Indonesia, Eijkman found that the beriberi developing in peoples eating polished rice could be prevented and cured by the feeding of an extract of rice polishings. Thus, a great amount of basic knowledge has built up from studies of the reasons for the geographic variations in human and animal diseases. There is no substitute for the shrewd guess in geographic pathology. The shrewd guess is the expression of genius. Walter Reed led a group of American Army doctors to Havana in 1900 and proved the mosquito transmission of yellow fever. Using human volunteers, he tested several hypotheses then current to explain the spread of yellow fever. Reed used systematic methods of investigation and has been credited with the discovery. But it was the Cuban physician, Carlos Juan Finlay, who in 1881, originated the shrewd guess that the mosquito, specifically the *Aedes aegypti* mosquito, transmits yellow fever. The investigative studies of these diseases: pellagra, scurvy, beriberi, and yellow fever—reveal the impact of geographic pathology on medical knowledge.

Geographic pathology, then, presents invaluable opportunities for exploration of the causes and pathogenesis of diseases of man through the techniques of epidemiology and modern research methods. Highly significant selective factors may be determined, and final proof of cause and effect may be obtained by appropriate ancillary studies. Such work may require an interdisciplinary approach involving numerous different talents. The investigators need to inquire into the hereditary, biologic, and environmental aspects in the lives of the individuals who comprise the contrasting populations.

CANCEROUS DISEASES OF GEOGRAPHIC IMPORTANCE

With regard to cancer, the world's literature on this subject contains numerous references to striking differences in its geographic distribution. Cancer is a frequent cause of illness in all races and ethnic groups and on all continents, subcontinents, peninsulas, islands, and archipelagoes. Moreover, the striking variations in the patterns of cancer that are now known to exist lend themselves admirably to investigative studies. Cutaneous cancers, so frequent on the face and hands in light-skinned people living, say, in Argentina, South Africa, and Australia, show a predilection for the legs and the soles of the feet of dark-skinned people living in tropical Africa and New Guinea. Cancer of the penis constitutes 30 percent of skin cancers in Vietnam. Kaposi's sarcoma alone equals the sum of all other types of skin cancers in the peoples of the North Central Congo. The incidence of cancer of the skin is particularly high in parts of Argentina and Taiwan where the people drink water contaminated with arsenic. Inbreeding among the inhabitants on the Cayman Islands, off Panama, and in the Minas Gerais District of Brazil has resulted in a high frequency of xeroderma pigmentosum with the inevitable accompani-

ment of multiple cutaneous cancers due to hypersensitivity to ultraviolet light. In the different segments of the alimentary tract—the mouth, oral pharynx, esophagus, stomach, and intestines—the racial and geographic variations in the incidence of cancer are so striking as to leave no doubt that cancer of each of these sites has special and particular etiologies yet to be disclosed. Opportunities for the investigation of high frequencies of alimentary tract cancers exist in different races and in different parts of the world: Oral cancer is frequent in India and Thailand; nasopharyngeal cancer in the Chinese in China, Taiwan, and Singapore and in Africans in Kenya; esophageal cancer in the Transkeian Bantus of South Africa, in women of northern Sweden, and in the cowboys of Southern Brazil; gastric cancer is frequent in Japan, Iceland, and Chile; intestinal cancer in North America and Europe. Equivalent or even more striking differences exist in the frequency of liver cancer. In one race alone, the Negro race, there are these marked geographic variations: Sixty percent of all cancers are cancers of the liver in Mozambique Negroes, whereas not more than 1 or 2 percent of cancers in USA Negroes are liver cancers.

The list of geographic differences in the frequencies of various cancers can be extended to cover virtually the entire field of neoplasia: cancers of the male and female genitourinary tract and sex organs, the exocrine and endocrine glands, the respiratory tract, the central and peripheral nervous system, and the hematopoietic tissues. The peculiar lymphoma of Tropical Africa, with its predilection for the jaws of 6 year olds, that has recently been called to the attention of the medical profession by the publications of O'Conor, Davies, and Burkitt, affords a unique challenge to investigators in geographic pathology. Peru, on the other hand, is admirably adapted for a study of the effects of elevation on the incidence and site distribution of cancers, since more than 12,500 feet of altitude separate the peoples living on the sea coast from those living on the shores of Lake Titicaca. Our host here in Lima, Dr. Pablo Mori-Chavez, is currently studying the effects of these physical differences on cancer and will present a paper on this subject at today's session.

SELECTIVE FACTORS AND RESEARCH

It was natural then, as well as clever, for Jacques May to define geographic pathology as: "Who has what, where, and why?" The aim of an epidemiological study is to identify all agents that impinge upon the individual—which might be associated with the existence or the occurrence of a disease. As clues emerge, they need testing in the laboratory. Laboratory investigation in geographic pathology lags behind today. It is often difficult to pursue. Experiments to elucidate etiologic factors involve originality of thought and physical facilities as well. Their pursuit may require the construction of laboratories in remote places where a given disease occurs frequently. Highly skilled investigators can then employ experimental methods to investigate the clues that the field studies have

revealed. The success of such enterprises depends largely upon the recruitment and retention of medical personnel in the critical areas of geographic and population differences.

TECHNIQUES

The disclosure of reliable information about the most prominent of the cancers that I have mentioned today would affect current attitudes regarding the relative importance of intrinsic and extrinsic factors. Reasonably accurate total incidence data are as yet available from only limited areas, which unfortunately do not always reflect the extremes of variation in environmental conditions. We therefore need to pursue rate studies vigorously. But while we may not soon acquire valid comprehensive statistics from some of the more critical communities, which unfortunately have limited medical facilities, we nevertheless can continue to study and to report upon the distribution of anatomical and histopathologic types of cancers in these communities. These reports will select places for intensive epidemiologic study and serve in the sense of a bioassay for the direction of such studies.

Sound methods for the study of geographic variations of the cancerous and other chronic diseases are a development chiefly of this century. Rapid advances in communication and travel have accelerated the application of these methods. Now known to be essential in a rate study are: 1) a defined geographic area within which the size, composition, and age and sex distribution of the population are known; 2) the collection of data on the disease and on the characteristics of the affected people as completely and accurately as possible; and 3) the use of comparable descriptive terms, means of diagnosis, and statistical methods. Actually, the census and survey methods employed in highly developed communities are often unsuited for underdeveloped communities. In particular, methods need to be devised for the statistical investigation of diseases in primitive peoples, such as the Bushman peoples of southwest Africa and the Kalahari Desert, the Aborigines of Australia, and certain Polynesian and Melanesian peoples. Investigators who accompany public health officers engaged in a program of mass vaccination can, at the same time, select those suspected of having cancer from among the persons who appear for vaccination. The suspects can then be referred to a hospital center for definitive diagnosis. Numerical estimates of populations can be calculated from representative samples. Prates employed aerial photographs to determine the size of the population in the native communities around the city of Lourenço Marques in preparation for a survey of cancer rates in Mozambique Africans. African natives live in scattered huts. Maps derived from the aerial photographs made possible the conduct of an accurate survey of the population structure.

In Africa, natives fix the seasons of the year by certain constellations. They take note when a fire has to be kindled at midnight or when direc-

tional shadows are different. The age of a patient, when unknown to himself, may be estimated by exploring his memory for a local event, often a catastrophic event, such as a war, fire, pestilence, famine, or drought. Dates are fixed by a total eclipse of the sun, the year in which the riots occurred, when the Chief married his great wife, when the floods came, or when the men returned from work in the city to supervise spring plowing and to celebrate with a continuous round of beer-drinking. Men often marry around the age of 25 years; the women around age 20; the firstborn usually arrives within a year of the marriage. It is possible, then, by different means to determine the age of individuals within 5-year age groups. For the collection of other information, methods that are closely allied to local conditions must often be devised.

RESUMEN

La investigación de cancer hasta ahora creo que ha enfocado en demasiá su atención en los pequeños animales de laboratorio, desperdiando las oportunidades que existen para estudiar el cancer humano en todas sus variadas manifestaciones. La experiencia enseña que las grandes contribuciones a la medicina y a la ciencia han surgido del trabajo cimentado sobre ideas que conciernen a la enfermedad humana. Es importante establecer y sostener este punto: el estudio de la enfermedad humana, en la que la naturaleza, y no el investigador, ha establecido las condiciones experimentales, constituye una investigación del más alto orden. La falta de dinero, de empresa y de interes, y la cautela y falta de imaginación de los altos administradores, son los responsables en cierta forma de este afrontoso relegamiento del hombre y de sus cánceres en favor del método mucho más fácil de estudio del cancer en los pequeños animales de laboratorio. Es de admirarse como puede suceder tal cosa si se reflexiona que la meta de las investigaciones médicas sobre cancer es conocer precisamente el cancer humano con la esperanza fundamental de establecer en una amplia escala el diagnóstico precoz, la cura y el control del cancer en los seres humanos. En alguna forma debe ponerse fin al prejuicio contra el estudio de los seres humanos con cancer y procurar que se canalicen los mayores esfuerzos investigativos en el campo de la patología geográfica.

Growth of Experimental Tumors at High Altitude^{1, 2, 3}

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SUMMARY

The experiments carried out since 1955 in the Laboratory for Cancer Research at Lima, Peru, to study the effect of natural high altitudes on neoplastic growth are reviewed. Several spontaneous and induced tumors, as well as transplantable tumors, of inbred strains of mice were studied, and the differences found between the high-altitude and the sea-level locations are briefly analyzed. A greater frequency of metastasis to the lungs and other organs was observed at high altitude than at sea level with both spontaneous and transplantable tumors. Studies with two transplantable tumors are presented: an hemangioendothelioma in

BALB/c mice, and #678 ascites carcinoma in C3H mice (cf Proc Amer Ass Cancer Res 3: 346, 1962). These tumors were transplanted in their respective host recipients, and the growth and spread of both tumors were compared at high altitude and at sea level. A greater frequency and extension of metastasis to the lungs, heart, lymph nodes, and spleen were found at high altitude than at sea level. The possible mechanism of this marked metastasis at high altitude is discussed, and the need for further work to study this problem with other techniques is emphasized.—Nat Cancer Inst Monogr 14: 309-331, 1964.

I FEEL so humble standing before this audience of outstanding scientists from Europe and North and South America in the field for which this conference was organized.

I remember that a very capable scientist in the field of cellular division, one of our distinguished guests, was reluctant to accept our invitation to participate because he did not feel competent enough to handle the topic proposed to him. I ask myself then, why, being merely a beginner in the broad field of experimental cancer research, I dare to bring a paper to this conference. There is only one possible justification or excuse for my

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³ Grateful recognition is given to the Cerro de Pasco Corporation for the local facilities that established the High Altitude Laboratory in Morococha, Peru.

⁴ Director, Laboratorio de Investigación de Cancer.

daring to do so, and this is that our good promoter of this conference, Dr. Hollaender, stated that this Symposium was mainly organized to stimulate basic research in the field. I certainly wish and I do hope that I will benefit from your criticism, comments, and suggestions regarding the observations that I now present.

HISTORICAL ACCOUNT

Studies of the effect of high altitude on the physiologic and pathologic processes have recently attracted much attention and have been of special importance in the progress of aviation. Most of these studies were carried on in the United States and in other countries where high altitudes have been simulated by use of pneumatic chambers at different levels of decompression or by gas mixtures to lower the oxygen tension. This experimental method is of great value in aviation studies in which man is temporarily exposed to the effect of high altitude.

Among the problems of environmental cancer and the geographic pathology of cancer, the effect of natural high altitudes is indeed of great importance. We do not yet have studies on geographic pathology of cancer of human populations living at high plateaus in the Andes. It was said, especially in the past, that cancer is a rare condition at high altitudes, and that leukemia almost never occurred. However, some recent, isolated observations on inhabitants at high altitudes of Peru, in the central and southern part of the Andes, have shown that occurrence of cancer of the genital tract in women is of high frequency, that cancer of the gastrointestinal tract occurs next in frequency, and that leukemia or lymphomas also occur. The extent of these frequencies or variations in comparison with other ethnic or geographic groups cannot be ascertained at the moment, and further studies on geographic pathology in human populations of the Andes should be undertaken.

We thought we might contribute some understanding to the problem of the effect of natural high altitudes on the growth of normal tissues and on growth of tumors, taking advantage of our unique geographic situation permitting us to study tumor growth at a high-altitude laboratory in Morococha, which is 14,900 feet above sea level, with a mean barometric pressure of 446 mm Hg, and travel time of about 4 hours from our sea-level laboratory in Lima.

GENERAL CONDITIONS OF THIS RESEARCH WORK

We began our experiments by raising an animal colony of several inbred strains of mice, such as the C58 for leukemia, C3H for mammary tumors and strain A for pulmonary tumors. These animals could not

breed at high altitude, so they were all bred at sea level and then similarly distributed between the two locations by littermate and sex at the time of weaning. Aside from the barometric pressure, the experimental and environmental conditions in the two laboratories, such as temperature, humidity, food and water supply, were kept as nearly alike as possible.

OBSERVATIONS

We first started a pilot study on the rate of mitosis in the epidermis of the ear of mice. It showed a marked depression during the first 3 days after exposure to high altitude hypoxia. A rapid recovery followed in the next few days that reached its highest activity at the 8th day, which seemed to be the time of release from initial restraint (1).

The healing of incised wounds in rats showed an accentuation of fibroplasia at high altitude in comparison to sea level, but the total duration of the healing process was the same at the two levels (2).

Our observations regarding tumor growth indicated a significantly lower incidence of spontaneous leukemia at high altitude than at sea level in the C58 mice (19.7% difference). The results also showed that leukemia developed at an earlier age at sea level than at high altitude. The incidence of leukemia in male mice was particularly higher at sea level than at high altitude. The anatomical lesions observed at high altitude in leukemia were not so extensive or prominent as those observed at sea level (3).

On the contrary, the incidence of spontaneous pulmonary tumors was greater at high altitude than at sea level, but the percentage difference was not statistically significant. However, the average diameter of the tumors at high altitude was greater than at sea level, and the difference was even greater between males at both levels. Associated with this increase in size, and evident both in male and female mice, was the greater occurrence of tumors of 4 mm and larger. Four mice at high altitude and 1 at sea level developed pulmonary tumors with anaplastic or sarcomatous characteristics (4).

No significant difference was seen in the incidence of spontaneous mammary tumors in strain C3H mice, but tumors appeared earlier at high altitude than at sea level. More interesting was the greater frequency of pulmonary metastasis at high altitude in mice bearing mammary tumors, which almost doubled that at sea level.

Other experiments with estrogen-induced leukemia and estrogen-induced mammary tumors were carried out by implanting 10 percent diethylstilbestrol-cholesterol pellets in the mice. Leukemia in the C58 estrogen-treated mice was again less frequent at high altitude than at sea level, but the percentage difference was not as significant as the spontaneous occurrence of this condition at either level.

The incidence of mammary tumors in C3H mice implanted with estrogen pellets was the same at high altitude and sea level. However, these

tumors metastasized more often at high altitude than at sea level, although the difference was not as great as in mice bearing spontaneous mammary tumors. An important observation in these C3H mice implanted with estrogen pellets was the occurrence of 11 pituitary tumors in the 168 mice kept at sea level, while none were found among the 181 mice kept at high altitude. The mice with pituitary tumors all had bilateral enlargement of the ovaries with stromal luteinization (5). Figures 1 and 2 illustrate these tumors.

Experiments on pulmonary tumors induced by urethan (ethyl carbamate) in strain A mice, given a single intraperitoneal injection of 0.25 mg, 0.5 mg, or 1 mg of urethan per g body weight, clearly indicated that high altitude, or factors related to altitude, enhanced the carcinogenic effect of urethan (6). At all three dose levels, a significantly greater number of tumors developed at high altitude than at sea level. The number of mice with large tumors (4 mm in diameter and larger) and the percentage of these large tumors were greater at high altitude than at sea level. Anaplastic changes were observed in 23 of 287 mice at high altitude and in 5 of 292 mice at sea level. Six tumors at high altitude and one at sea level infiltrated the adjacent structures (fig. 3).

OBSERVATIONS ON METASTASIS

We studied two transplantable tumors: 1) a hemangioendothelioma of BALB/c mice, kindly provided by Dr. Richard Swarm of the National Cancer Institute, National Institutes of Health, Bethesda, Maryland. The original tumor developed in the spleen of a BALB/cAnN mouse following intravenous administration of colloidal thorium dioxide and is nearly identical to similar tumors produced in rabbits (7); 2) #678 ascites carcinoma of C3H mice developed in our laboratory.

Hemangioendothelioma of BALB/c mice

This tumor grew in 100 percent of the hosts upon subcutaneous transplantation into BALB/c mice and also into (BALB/c \times A)F₁ hybrid mice. On gross examination, the tumor was very vascular and rather soft, with large cystlike spaces filled with blood. We had a group of 62 BALB/c mice that had received tumor transplants when they were 16 weeks old and air-shipped to Lima by Dr. Swarm. They were equally distributed between the high-altitude and the sea-level locations. The tumors were first palpable 3 weeks later, having a variable growth, averaging 2.5 cm in size and 1.5 g in weight when the mice died, or were killed when about to die, at an average age of 11 weeks after transplantation. The mice showed hypervolemia at the beginning, and finally a marked anemic condition. Table 1 shows no important difference in the growth of this tumor between the high-altitude and the sea-level locations.

TABLE 1.— Transplantation experiments with a hemangioendothelioma of BALB/c mice at high altitude

Experimental group	Num- ber of mice	Age (weeks)			Weights (g)		
		At tumor trans- plant	Tumor pal- pable	At death	Tumor	Organs	Animal
High altitude (Morococha) 14,900 feet	31	16	19.1	27.1	1.5	3.60	25.4
Sea level (Lima)	31	16	19.8	27.2	1.3	3.45	26.1
Differences			-0.7	-0.1	+0.2	+0.15	-0.7

The distribution of metastatic growths is shown in table 2. Figures 4 through 9 illustrate the pathological findings at both locations, which demonstrate that the tumor was a highly metastasizing one. Its spread in order of frequency at high altitude was to the following sites: lungs, spleen, subcutis, peritoneum, and muscle; at sea level, its spread was to lungs, peritoneum, spleen, subcutis, and muscle. The differences in the incidence of metastasis to the spleen, subcutis, lungs, and muscle were, respectively, 51.6, 25.8, 21.6, 18.7, and 16.6 percent greater at high altitude than at sea level. Only the frequency of peritoneal implants was reversed, being 12.9 percent greater at sea level than at high altitude.

Marked extramedullary erythropoiesis was observed in the spleen of mice bearing the hemangioendothelioma, especially in those instances in which the spleen was the site of metastasis. This erythropoiesis was 33.3 percent more significant at high altitude than at sea level.

Transplantation of this hemangioendothelioma from BALB/c to (BALB/c \times A)F₁ hybrids again showed a 100 percent of "takes," but the frequency of metastasis was reduced at both locations and mostly limited to the lungs, with 66.7 percent at high altitude and 38.9 percent at sea level. The transplantation experiments were then continued from F₁ to F₂ hybrids, and there was a greater reduction in the metastasizing ability of this tumor—19.5 percent at high altitude and 9.1 percent at sea level. When the BALB/c mice in our colony were available, the tumor was transplanted from the F₁ hybrids back to the BALB/c mice. Again we observed the 100 percent of "takes," but the metastasizing ability of the tumor practically disappeared.

No gross or microscopic differences in this tumor were observed at high altitude in comparison with its characteristics at sea level.

It was evident that the frequency of metastatic growths of this hemangioendothelioma and also the size and extent of the metastatic nodules were greater at high altitude than at sea level.

#678 Ascites Carcinoma in C3H Mice

In 1956, this tumor was found growing in the stomach of a female C3H mouse and spreading out to the omentum and mesentery with hemorrhagic ascitic fluid [(8) and fig. 1].

TABLE 2.—Transplantation experiments with a hemangioendothelioma of BALB/c mice—distribution of metastatic growths

Experimental group	Lung		Spleen		Peritoneum		Muscle		Subcutis		Erythropoiesis	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
High altitude (Moroccocha) 14,900 feet	30	96.8	23	74.2	10	32.2	8	25.2	14	45.1	27	87.1
Sea level (Lima)	23	74.2	7	22.6	14	45.1	2	6.5	6	19.3	17	54.8
Differences	+7	21.6	+16	51.6	-4	12.9	+6	18.7	+8	25.8	+10	33.3

Histologically, this tumor was a solid squamous cell carcinoma (fig. 11). It was transplanted both subcutaneously by the trocar method and intraperitoneally by an emulsion of the tumor. After several intraperitoneal passages, the tumor was progressively converted to the pure ascites form that killed the mouse in 7 to 8 days. At present the tumor has had more than 300 transplant passages.

Paralleling the conversion to the ascites form, a progressive change in the histologic pattern from the differentiated to the undifferentiated squamous cell carcinoma was observed (figs. 12 and 13). When this tumor was transplanted subcutaneously with 0.10 ml of ascitic fluid, it grew in 100 percent of the host mice to large solid tumors that reached more than 3 cm in diameter and killed the mouse in about a month. The tumor showed highly metastasizing ability to the regional lymph nodes, lungs, and heart (figs. 14 through 21).

Table 3 shows the percentage frequency and the distribution of metastatic growths of this tumor in groups of mice at high altitude and at sea level. Each group received subcutaneous transplants of a saline dilution of ascitic fluid containing 400,000 tumor cells. The mice were killed at intervals of 30, 25, and 20 days after transplantation.

TABLE 3.—Over-all frequency of metastasis of #678 ascites carcinoma in C3H mice at high altitude and at sea level

Experimental groups		Frequency of metastases			
Days*	Location	Number of mice	Tumor weight	Number of mice	Percent \pm SE
20	High altitude	50	2.26	28	56.0 \pm 7.02
	Sea level	51	1.83	16	31.4 \pm 6.49
	Difference		0.43		24.6 \pm 9.55
25	High altitude	75	4.46	63	84.0 \pm 4.20
	Sea level	87	5.27	67	77.0 \pm 4.51
	Difference		-0.81		7.0 \pm 6.16
30	High altitude	152	5.77	144	94.7 \pm 1.82
	Sea level	147	5.53	123	83.7 \pm 3.05
	Difference		0.24		11.0 \pm 3.54

*Days after subcutaneous transplantation.

No significant difference in the subcutaneous tumor growth between the high-altitude and sea-level locations was found. The tumors grew to the same size and averaged about the same weight. No histological change or difference could be observed between the two levels.

The percentage frequency of metastatic growths in mice bearing tumors was great in the 30-day groups following transplantation and small in the 20-day groups, regardless of location, which indicates that the time factor is important in the development of metastatic growths as shown previously by others (9). In all three groups, there was a greater percentage frequency of metastatic growths at high altitude, and this increase

was much more significant when considering the 20-day groups in which there was a percentage difference of 24.6 ± 9.5 , favoring the high-altitude mice. This indicated that metastasis occurred earlier at high altitude than at sea level.

The percentage distribution of metastatic growths in the lymph nodes, lungs, and heart, shown in table 4, is referred to the number of mice bearing metastatic growths at any or to all sites indicated.

TABLE 4.—Distribution of metastatic growths of #678 ascites carcinoma in C3H mice at high altitude and at sea level

Experimental groups		Number of mice	Distribution of metastases (percent \pm SE)		
Days*	Location		Lymph nodes	Lungs	Heart
20	High altitude	28	17.8 \pm 7.22	85.7 \pm 6.61	3.6 \pm 3.52
	Sea level	16	50.0 \pm 12.50	50.0 \pm 12.50	—
	Difference		32.2 \pm 14.43	35.7 \pm 14.14	—
25	High altitude	63	77.8 \pm 5.23	92.1 \pm 3.39	11.1 \pm 3.95
	Sea level	67	77.6 \pm 5.09	73.1 \pm 5.41	3.0 \pm 2.08
	Difference		0.2 \pm 7.29	19.0 \pm 6.38	7.9 \pm 4.46
30	High altitude	144	69.4 \pm 3.84	95.1 \pm 1.79	22.2 \pm 3.46
	Sea level	123	56.1 \pm 4.47	83.7 \pm 3.33	7.3 \pm 2.36
	Difference		13.3 \pm 5.89	11.4 \pm 3.78	14.9 \pm 4.41

*Days after subcutaneous transplantation.

In an analysis of metastases to the lungs, it is clearly shown that the increase is greater in the mice kept at high altitude than in those at sea level. The difference between the three groups was even greater when the time following transplantation was shorter. The percentage differences in the 30-day, 25-day, and 20-day groups were respectively, 11.4 ± 3.78 , 19.0 ± 6.38 , and 35.7 ± 14.14 greater at high altitude than at sea level.

The extent of metastatic growth to the lungs and heart was much greater at high altitude than at sea level. This growth could not be evaluated by counting and measuring the tumor nodules because very often the infiltration was so extensive that the nodules could not be counted (fig. 18).

The differences in the lymph node metastases were not very important among the 30-day groups and were even reversed in the 20-day groups, showing a 32.2 percent greater difference at sea level than at high altitude.

The metastatic growths to the heart (table 4) are also much greater at high altitude than at sea level. The percentage differences found between the 30-, 25-, and 20-day groups were 14.9, 8.3, and 3.6. At both locations, the frequency of metastatic growths to the heart is directly related to the time that elapsed following transplantation.

Other experiments are under way to study the metastasis at high altitude by intravenous injections of the same #678 ascites carcinoma in

C3H mice. The results clearly indicate that the frequency of metastasis, especially to the lungs, and the number of tumor nodules per mouse, is much greater at high altitude than at sea level. The seeding and growth of the tumor cells were established much earlier at high altitude than at sea level.

DISCUSSION

The observations we have presented here regarding tumor growth in inbred strains of mice at high altitude and at sea level clearly indicate that exposure to *natural* high altitude at Morococha, 14,900 feet above sea level, produced changes in the growth of certain types of tumors, but not in others. The incidence of leukemia was lower at high altitude than at sea level, whereas spontaneous pulmonary tumors were not only more frequent at high altitude but also grew to larger size than at sea level; urethan evoked more lung tumors at high altitude than at sea level. We have observed 11 pituitary tumors associated with bilateral ovarian luteomas among the estrogen-treated C3H mice at sea level but none at high altitude.

The results of studies in decompression chambers at *simulated* high altitudes were different. Some isolated reports have intermittently appeared since the original work of Warburg, in 1926, which indicated that simulated low oxygen tension inhibited the growth of tumors or induced necrosis in tumors already grown (10-14). Most of these studies were carried out in pneumatic chambers at different levels of decompression, or by use of gas mixtures to lower the partial pressure of oxygen from 360 to 300 mm Hg. We believe that such simulated altitudes do not truly reproduce the conditions of natural acclimatization. The animal kept in a tank at such high altitudes is not responding to the effect of anoxia stimulation, but rather to anoxia deterioration.

From the research work done at the Peruvian Medical School, begun by Professor Mongé and followed by Professor Hurtado and his co-workers, we know that there are important anatomical and physiological differences between the native resident at high altitude and the man at sea level. Hurtado suggested that the adaptation mechanism is related to oxygen utilization and energy production at the tissue level, and it is believed that the tissue adjustments may contribute the fundamental characteristics of natural acclimatization (15). It is obvious that the endocrine and the neuro-vegetative systems play an important role in the tissue changes, as the *primum movens* in the adaptative process to the high altitude environment (16, 17).

We do not intend to discuss or to speculate on the mechanism of these changes at high altitude in spontaneous, induced, or transplantable tumors and their metastatic growths. But we would like to analyze briefly some factors that may be desirable to investigate in regard to the increased frequency of metastatic growths observed at high altitude.

One of the properties of malignant cells is their ability to spread or metastasize to distant organs. The presence of metastasis is probably

the most important single factor in the prognosis of a tumor. There is still little known about the conditions that determine the spread of tumors. According to Willis, the invasive properties of tumors reside largely in the tumor cell itself (18). Coman stated that the local invasiveness of cancer cells is primarily dependent upon loss of their mutual adhesiveness (19-21), which is associated with or due to local calcium deficiency (22). The easily detached cancer cells are actively ameboid and are thereby able to penetrate the surrounding normal tissues, the lymphatic system, and the blood stream (23).

The fate of emboli of tumor cells may depend on chemical, metabolic, and endocrine factors, as well as on the immune tissue reaction at the site of the embolus, or may depend also on a general humoral condition of the organism.

There are probably mechanical factors involved in the arrest of tumor emboli in different organs and especially in the lungs, since they are continuously expanding and contracting, producing changes in pressure that affect the circulation in the lung.

How and to what extent these factors are involved in the increase of metastases observed at high altitude are questions which will be the subject of new research studies that we are planning. These will include application of methods to evaluate the rate of cellular division of transplantable tumors and their metastases; autoradiographic methods with tritium-labeled amino acids as radioactive precursors to deoxyribonucleic acid and ribonucleic acid; histochemical methods to study changes in the cell membrane and in the cementing substance; electron microscope studies to visualize any change or alteration in the cell surface or in other cell structures; and studies on the chemistry of tumors, especially enzyme activity at high and low altitudes. Some of these studies are under way, while others depend on extending research facilities and increasing personnel. Collaborative work on these several approaches, from abroad or locally, will be welcomed.

RESUMEN

Se hace una revisión de los experimentos realizados en el Laboratorio de Investigación de Cáncer en Lima, desde el año 1955, para estudiar el efecto de las grandes alturas naturales en el crecimiento neoplásico. Se han estudiado varios tumores espontáneos e inducidos y también tumores transplantables en cepas puras de ratones, y las diferencias encontradas entre la altura y el nivel del mar son analizadas.

Se ha observado una mayor frecuencia de metástasis en los pulmones y otros órganos o tejidos en la altura que al nivel del mar, tanto en tumores espontáneos como en los transplantados.

Se presentan los resultados obtenidos con dos tumores transplantables: Un hemangiointelioma de los ratones BALB/c, y el 678 carcinoma ascítico de los ratones C3H (*Ref Proc Am Ass Cancer Res 3: 346, 1962*). Dichos tumores fueron transplantados, en sus respectivos huéspedes recipientes, y se estudió luego, comparativamente, el crecimiento de los tumores y su difusión metastásica en la altura y al nivel del mar. Se encontró una mayor frecuencia y extensión de los nódulos metastásicos en los pulmones, ganglios linfáticos, corazón y el bazo en la altura que al nivel del mar.

Se discuten los factores o mecanismos probables de este aumento en la altura, y se recalca la necesidad de estudiar con otros métodos este interesante problema de las metástasis.

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PLATE 15

FIGURE 1.—A large pituitary tumor with bilateral ovarian luteomas in a C3H mouse implanted with diethylstilbestrol-cholesterol pellets at sea level.

FIGURE 2.—Another large pituitary tumor, dark because of extensive vascularization, in a C3H female mouse implanted with diethylstilbestrol-cholesterol pellets at sea level.

FIGURE 3.—Strain A mouse killed 59 weeks after a single urethan treatment at high altitude. Large pulmonary tumors with infiltration to the heart and chest wall, indicating a malignant change.

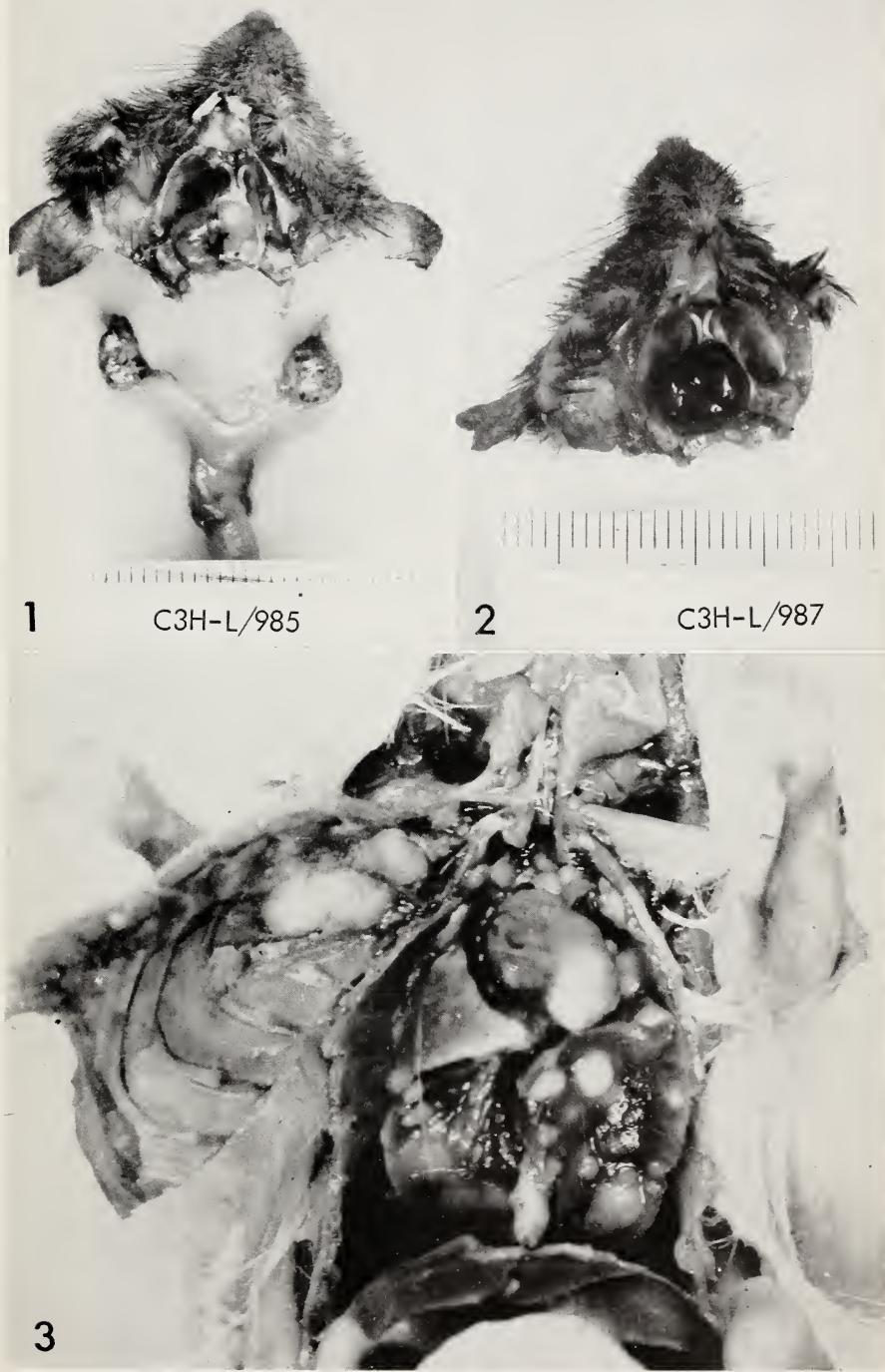


PLATE 16

FIGURE 4.—A BALB/c mouse transplanted with hemangioendothelioma at high altitude. Subcutaneous transplant on right side. Several metastatic growths in lungs, chest wall, and subcutis. Spleen is enlarged.

FIGURE 5.—Another BALB/c mouse transplanted with hemangioendothelioma at high altitude. The subcutaneous transplant on right side. Metastatic spread to lungs. Spleen is greatly enlarged with metastatic growth in its inferior end.

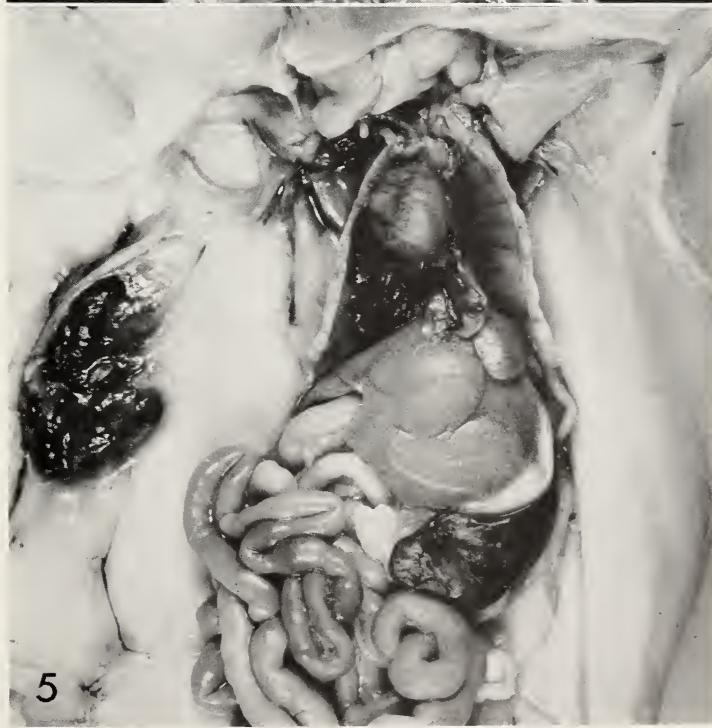


PLATE 17

FIGURE 6.—Microscopic section of hemangioendothelioma. On *left* is a rather solid part of the tumor composed of anastomosing vascular channels lined by endothelial tumor cells. On *right* is a cystic-like space with projections of tumor cells, and blood in between. Hematoxylin and eosin. $\times 300$

FIGURE 7.—Microscopic section of lung. Next to a large bronchus is shown metastasizing hemangioendothelioma. A large cystlike space is crossed by sheets of tumor cells. Hematoxylin and eosin. $\times 108$

FIGURE 8.—Microscopic section of spleen, showing huge metastatic growth of hemangioendothelioma, with a large cystlike area in which are growing anastomosing sheets of tumor cells in an areolar fashion. Hematoxylin and eosin. $\times 50$

FIGURE 9.—Microscopic section of heart, showing a metastatic growth below the epicardium. Hematoxylin and eosin. $\times 80$

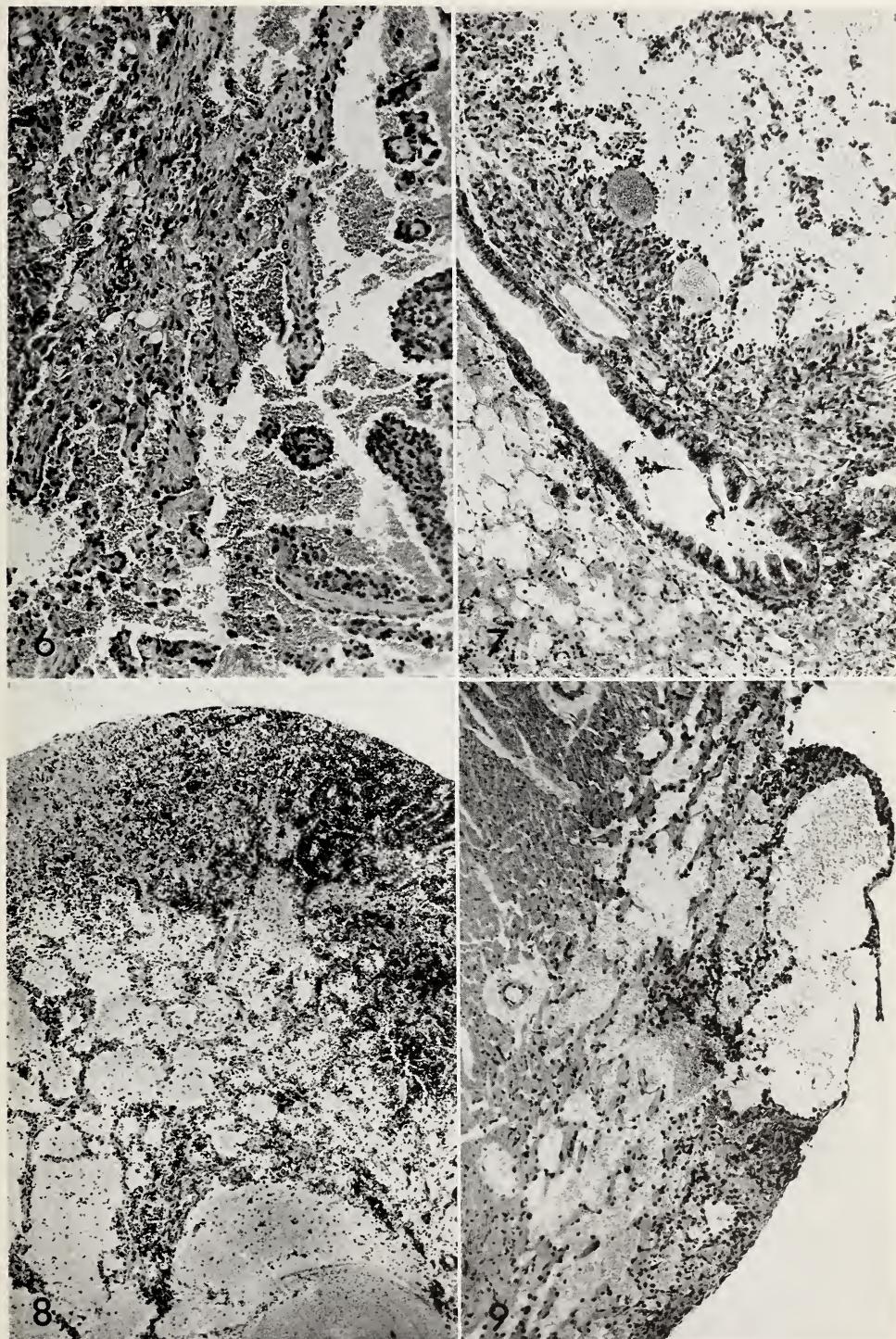


PLATE 18

FIGURE 10.—Original #678 ascites carcinoma of forestomach of a C3H female mouse. A large nodular tumor mass involves the stomach and disseminates to omentum, mesentery, and abdominal lymph nodes.

FIGURE 11.—Microscopic section of original tumor. Underneath normal squamous cell epithelium of forestomach can be seen extensive infiltration of submucosa with differentiated squamous cell carcinoma with epithelial pearls. Hematoxylin and eosin. $\times 15$

FIGURE 12.—Microscopic section of omentum of a mouse after 24 hours of intra-peritoneal transplant with #678 ascites carcinoma (118th transplant passage). Tumor cells loosely attached are implanting the surface of the omentum. Hematoxylin and eosin. $\times 240$

FIGURE 13.—Smear of ascitic fluid of #678 ascites carcinoma (7 days after transplantation) showing tumor cells with dense hyperchromatic nuclei, and several mitotic figures. Shorr's stain. $\times 480$

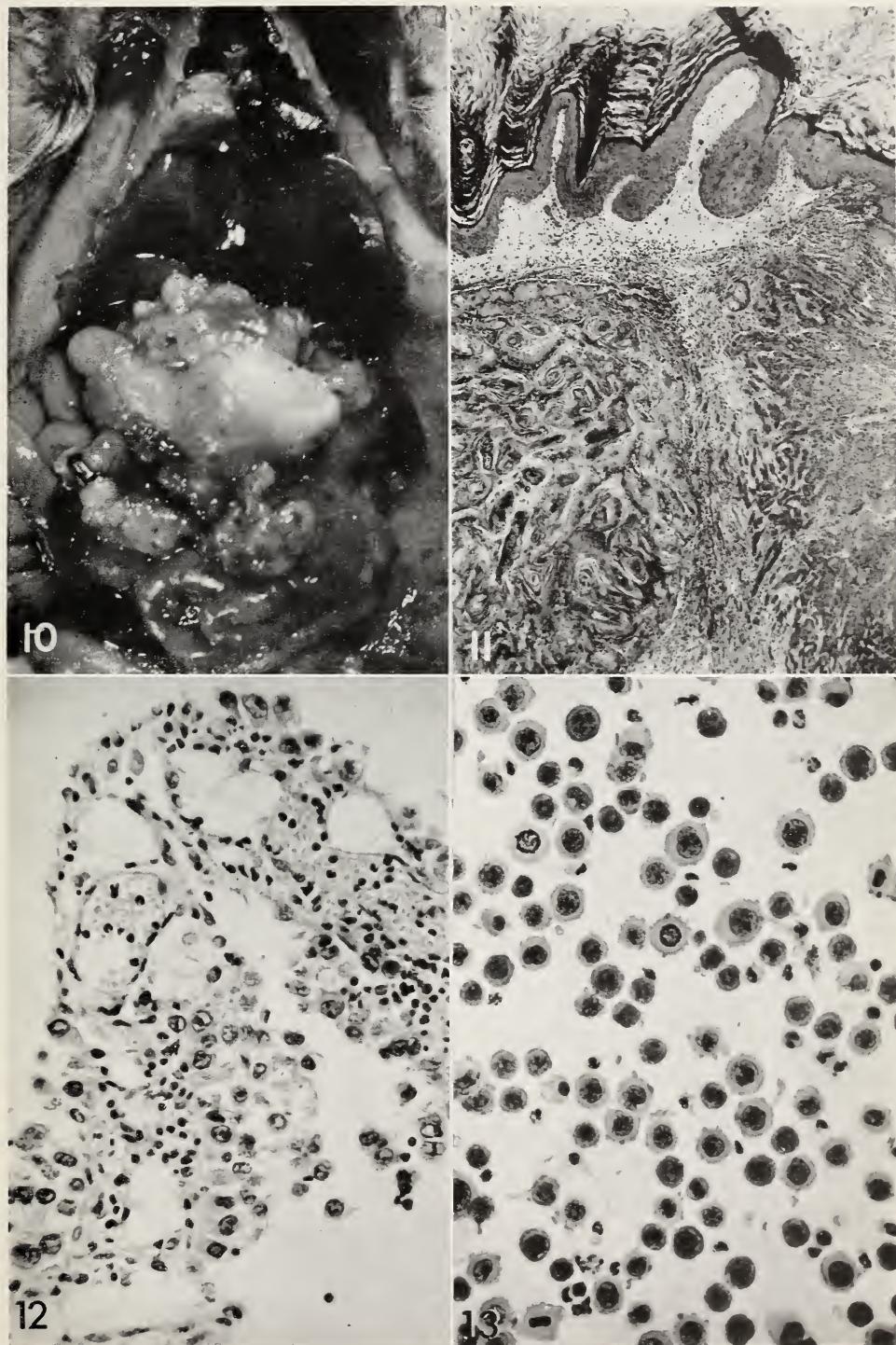


PLATE 19

FIGURE 14.—A C3H mouse subcutaneously transplanted with #678 ascites carcinoma at high altitude. A large lobular tumor mass on *right* has metastasized to the regional lymph nodes and to lungs with large tumor nodules.

FIGURE 15.—Photograph of lungs and heart removed from 5 C3H mice, subcutaneously transplanted with #678 ascites carcinoma at high altitude. Metastatic tumor nodules in lungs and/or in hearts are clearly shown.

FIGURE 16.—Microscopic section of the subcutaneous transplant of #678 ascites carcinoma. Undifferentiated carcinoma composed of cells with ill-defined borders grouped in a disorderly manner. Tumor cells show large, irregular, round nuclei with 1 or 2 nucleoli, and several mitotic figures. Hematoxylin and eosin. $\times 610$

FIGURE 17.—Microscopic section of lung with a metastatic tumor nodule. Hematoxylin and eosin. $\times 240$

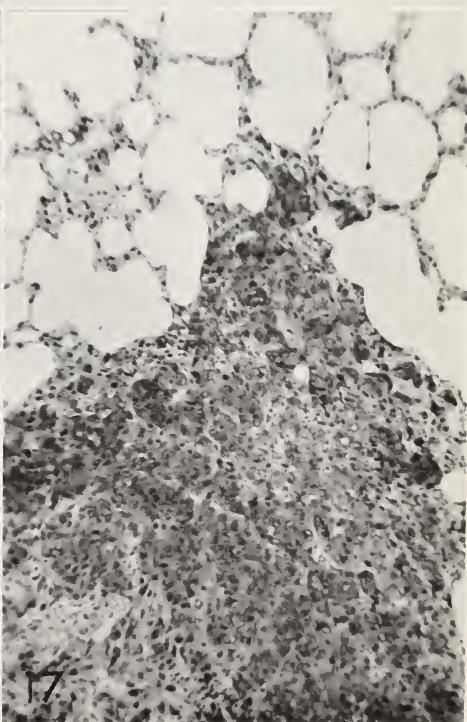
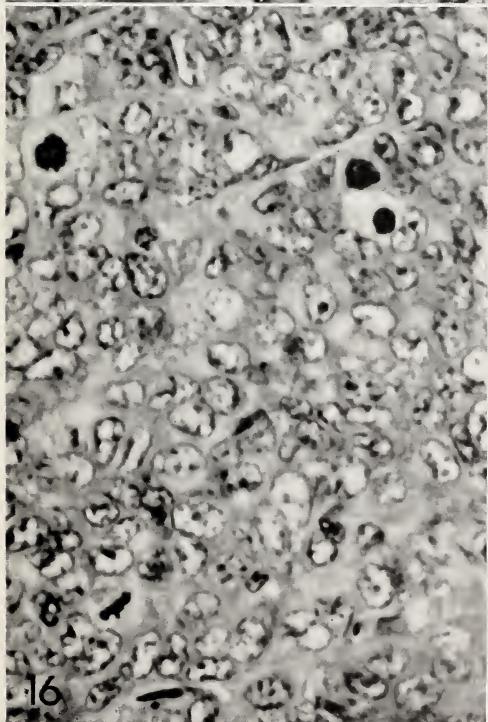


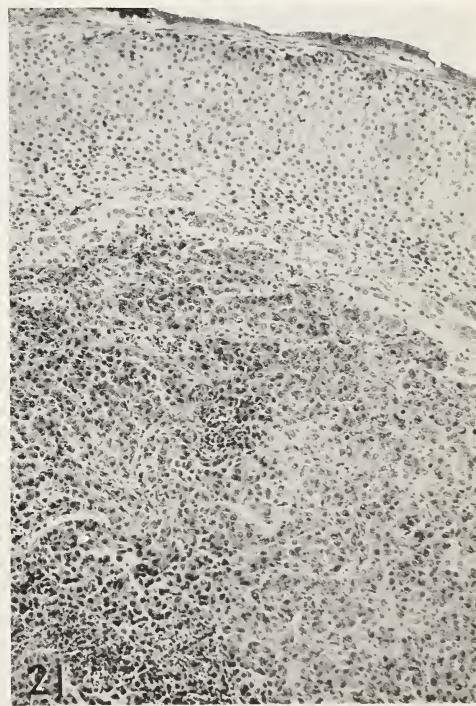
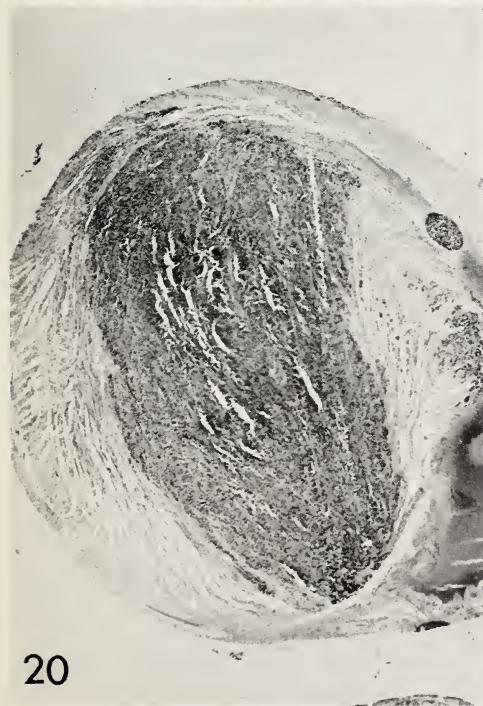
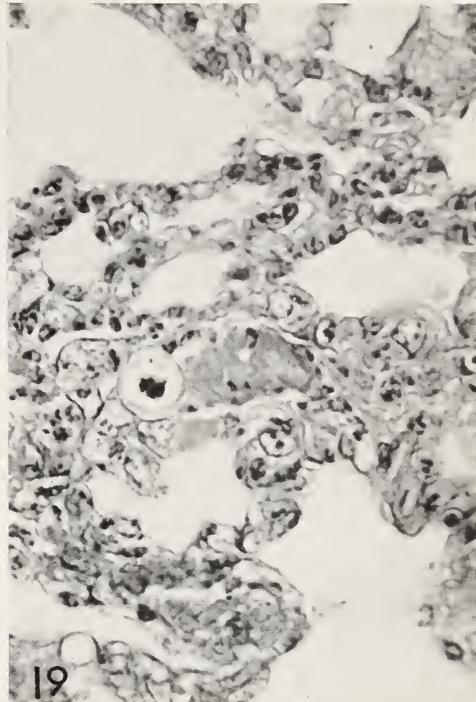
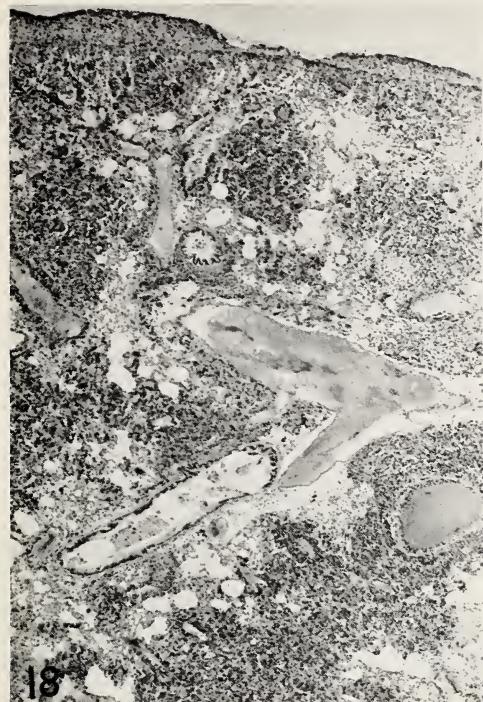
PLATE 20

FIGURE 18.—Microscopic section of lung of a C3H mouse killed 30 days after subcutaneous transplant of #678 ascites carcinoma. It shows a frequent type of huge or diffuse spread of metastatic growth at high altitude. Hematoxylin and eosin. $\times 35$

FIGURE 19.—Microscopic section of lung of a C3H mouse killed 25 days after subcutaneous transplant of #678 ascites carcinoma. Capillaries are filled with tumor cells. A dividing cell is clearly shown. Periodic acid-Schiff. $\times 475$

FIGURE 20.—Microscopic cross section of heart showing ventricle occluded by a metastatic tumor mass. Hematoxylin and eosin. $\times 65$

FIGURE 21.—Microscopic section of adrenal gland of a C3H mouse killed 4 weeks after subcutaneous transplant of #678 ascites carcinoma. A large metastatic tumor mass is growing in medulla. Cortex is still preserved. Hematoxylin and eosin. $\times 108$



A Survey of Human Cancer at High Altitude in the Peruvian Andes¹

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SUMMARY

Contrary to popular belief, the present survey indicates that human cancer occurred at high altitude in the Peruvian Andes. A very high proportion of uterine cancer, usually in the cervix, and of cancer of the stomach was observed in women. The most common malignancies observed in men, in order of

frequency, were cancer of the stomach, lymphoma and leukemia, and pulmonary cancer. These malignancies were also reported to be unusually frequent in the Peruvian cities at or near sea level.—Nat Cancer Inst Monogr 14: 333-337, 1964.

DATA were collected for 10 years (1951-60) from the clinical records of the Chulec Hospital, Oroya, Peru, situated at an altitude of 13,000 feet. This hospital provides medical care to the employees, and their families, of an American mining company, the Cerro de Pasco Corporation. A crude estimate of the population served, from Oroya, Morococha, and Cerro de Pasco, all at 13,000 to 15,000 feet elevation, is 60,000 persons. These are almost entirely of Indian and mixed Indian descent.

The hospital is well equipped and its staff includes specialists in thoracic and abdominal surgery and in neurosurgery. A pathologist examines at least 90 percent of the tissues obtained at operation and necropsy. From 1951 through 1960, 32,834 patients were admitted. Malignant tumors were diagnosed in 217 patients. Tables 1 and 2 show the distribution of women and men by the site of their malignant tumor and the age at diagnosis. Not shown are 48 patients with diagnoses of benign tumor.

Rates of cancer, the most reliable measures for comparisons, were not calculated. The total population in the high-altitude area is poorly defined, due to lack of data on distribution by sex and age and because of migration. Furthermore, patients with inoperable cancers, especially of the uterus, were not admitted to the hospital and thus the survey data are incomplete.

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

Relative frequency calculations are less reliable, but they nevertheless identify the most frequent cancers. Uterine cancer, usually in the cervix, was 55 percent of all cancer in women at the Chulec Hospital, a very high proportion. Cancer of the stomach, 9 percent, was the next in order of frequency. The three most frequent cancers in the men living at high altitudes were: stomach, 28 percent; lymphoma and leukemia, 18 percent; and lung, 13 percent. Caceres has reported relative frequencies of cancer

TABLE 1.—Malignant tumors in women

Site or type of tumor*	Number of cases in age groups:						Total
	0-19	20-29	30-39	40-49	50-59	60 and over	
Uterus	5	35	31	13	3	14	87
Stomach			4	4	6	6	14
Intestine		1		2	3		6
Lymphoma and leukemia	1	3	1	2			7
Lung			1	2			3
Breast			3	1	1		5
Vagina and vulva		1	1		1		3
Ovary	1		1	2			4
Thyroid gland			2		1		3
Liver		1					1
Gallbladder and bile duct			1	2			3
Larynx and trachea				1	1		2
Skin					1	1	2
Kidney				1			1
Salivary gland		1					1
Chorioepithelioma				1			1
Wilms' teratoma	1			1			1
Retinoblastoma							1
Melanoma				3	1	2	6
Sarcoma, all sites	1	3		2			6
							157

*Tumors were carcinomas unless otherwise specified.

TABLE 2.—Malignant tumors in men

Site or type of tumor*	Number of cases in age groups:						Total
	0-19	20-29	30-39	40-49	50-59	60 and over	
Stomach				3	10	4	17
Intestine		1	1			1	3
Lymphoma and leukemia	4	2		1	4		11
Lung				2	3	3	8
Maxillary sinus				1		2	3
Mouth			1	1			2
Prostate gland					2		2
Pancreas			1				1
Liver					1		1
Thyroid gland			1				1
Seminoma			1				1
Meningioma						1	1
Melanoma				1	1		2
Sarcoma, all sites	2	1	1	2	1		7
							60

*Tumors were carcinomas unless otherwise specified.

calculated from data collected from Peruvian cities (1). Although these data were derived mainly from populations living at or near sea level, the same cancers were unusually frequent as we have described for the high-altitude area.

RESUMEN

Este informe indica que el cancer humano se observa en las grandes alturas de los Andes peruanos. Una alta proporción de cancer uterino, comunmente en el cuello y luego cancer del estómago se observan en la mujer. Los canceres más comunes que se observan en el hombre son, en orden de frecuencia, cancer del estómago, linfoma y leucemia, y cancer pulmonar. Estos mismos canceres se han referido como muy frecuentes en ciudades del Perú al nivel del mar ó a bajas alturas.

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DISCUSSION

Tannenbaum: The work of Mori-Chavez is important because it adds further indirect evidence that cancer occurs in all populations. In earlier years there were myths regarding the lack of development of neoplasms in American Indians and native Africans south of the Sahara. Now it is well known that this is not true; the concept probably arose through inadequate observations and paucity of autopsies, and short lifespans. In all likelihood, these same factors resulted in the general viewpoint that cancer developed in far lesser incidence among native Peruvians living at high altitudes than among those living at lower altitudes.

In extensive and carefully designed experiments with mice, in which one group was housed at sea level and the second at a high altitude, Mori-Chavez has studied the comparative occurrence of a number of types of tumors. The fact that tumors arose in both groups is more significant, in my opinion, than the small differences found with certain tumor types.

The finding that there is a considerable differential metastasis rate between mice kept at low and at high altitudes is intriguing. Perhaps this observation may turn out to have even more significance than Mori-Chavez's basic observation that tumors do arise in animals kept at high altitudes. It may open the way to experiments that could help to elucidate the factors and mechanisms involved in the spread and metastases of neoplasms. This is probably one of the most important fields in cancer research, too long underemphasized and neglected. I do hope that Dr. Mori-Chavez will continue and expand these investigations. Congratulations for excellent research accomplished under difficult conditions.

Mori-Chavez: I sincerely appreciate Dr. Tannenbaum's stimulating comment on my research work. I benefited myself from his constant help and advice. He warned me at the start of my experiments that I should be very careful about the nutritional condition of the animals. I followed this good advice and gave the same water and food, and established similar conditions at both levels. I have noted the animals eat more at high altitude, but the weight gain was less than at sea level. It is worth mentioning that in spite of this difference in weights favoring the mice at sea level, those kept at high altitude with lower average weight had more pulmonary tumors.

Hueper: Dr. Mori-Chavez, have you studied the vascular system? Because of the high incidence of arteriosclerosis under anoxic conditions, one would expect that there

should be vascular damage in mice kept at a high altitude. The existence of such relations was demonstrated recently by investigations at the National Institutes of Health where dogs were kept at an artificial altitude of about 14,000 feet. At autopsy, distinct arteriosclerosis was present which was not found in controls kept at sea level. I would expect that the colonization of tumor cells at sites of damage to the endothelial lining would be favored, and that metastatic cells would survive in the lungs. Therefore, we would have a higher distribution and a higher incidence of metastatic foci in the mice kept at high altitude than in those kept at sea level.

Mori-Chavez: I think this is an important question; the role of vascular changes. What we know from study of man at high altitude is that high blood pressure is a rare condition in comparison to man of sea level and that coronary thrombosis and myocardial infarct rarely occur also. We have not made especial studies regarding arteriosclerosis and other vascular diseases in mice and rats at high altitude.

Upton: I wonder whether polycythemia, which I should have expected to occur at high altitude, might have caused vascular congestion and stasis which could have favored the settling and seeding of the test tumor cells. Much has been made of the regular occurrence of tumor cells in the circulatory system, and perhaps congestion and stasis might account for the greater vascular metastases or for the greater invasiveness of tumors in mice at high altitudes. I would be interested, Dr. Mori-Chavez, to know what the red cell levels were in the high-altitude mice and whether you think these animals had hypervolemia.

Another point you might possibly consider in studying the invasiveness of tumors at high altitude is the application of the methods that Dr. Leblond so nicely demonstrated in his paper. For example, you might examine some of the tissues that appear to be behaving differently in your experimental material (e.g., the lung) for differences in the nuclear addition rate in relation to mitosis or cell turnover.

Mori-Chavez: Polycythemia, a very well-known condition in man for many years, is probably the main response to high altitude. This is of the absolute type with an increase in the total blood and red cell volumes, a decrease in the plasma volume, an elevated total circulating hemoglobin, and elevated blood viscosity. These changes were also observed in mice at high altitude. A capillary dilatation, and possibly an increase in the capillary bed of the tissues are, among others, compensatory adjustments observed in man and in the animals acclimatized to high altitude. I think some of these conditions may account, among other factors, for the increased metastatic spread of tumors at high altitude.

Regarding studies on the cell turnover, we have not been able to do such experiments as yet. We certainly agree that they may contribute useful information to the problem of neoplastic growth at high altitude.

Goodman: Something related to Dr. Upton's statement on circulatory physiology is whether the same number of cells might be free in both places: that just simply by virtue of the larger blood volume and a greater circulation, there would be a greater opportunity for metastases to occur. Some parabiosis experiments that might be done between mice with and without tumors, one experiment at low altitude and another at high altitude, to find out to what extent the nature of the circulating cell itself is involved in the metastasis, and to what extent local conditions, including oxygen availability, are involved.

Mori-Chavez: It is a very nice suggestion and I will try in the near future to do one experiment with parabiosis to see if there is this possibility.

Kaplan: I was also particularly intrigued by the work on metastasis, and would certainly support Dr. Tannenbaum's suggestion that this aspect of the work is perhaps the most important and most deserving of concentrated effort in the future. Certain other suggestions might be offered. It is true that the animals adapt to the high altitude, but I wonder whether quantitation of the adaptation might not be revealing. It seems to me that the mouse and other small rodents are already using just about every cubic millimeter of space in their bone marrow to make cells, even at sea level, and they usually have very little reserve volume for adaptation. It may be that

when the mouse is fully adapted at high altitude, he is less well adapted than a human being; humans have a much greater reserve volume of relatively unused bone marrow for production of additional red cells. Thus, it is possible that the adapted mouse at high altitude is relatively hypoxic. If this is really the case, then it would strongly suggest that the lack of oxygen may influence the behavior of tumor cells. This is not an unattractive hypothesis, as you have already mentioned, and it could affect metastasis in at least two ways: one is the likelihood that tumor cells would get into the blood stream in the first place, and the second is the likelihood that they would lodge successfully afterward.

I think it would be interesting to see whether some simple system could be devised for having a part of your animals (with various tumors) on 95 percent oxygen at high altitude, so their tissues and tumors could be brought up to full oxygenation despite the high altitude. If the oxygenation factor were the only cause of the increased metastasis you observe, I would expect that this would effectively suppress the metastatic frequency to the sea level value. It is also important to find out whether the effect is due to more tumor cells getting into the blood stream or the more successful implantation of those that do get in. One way to attack this problem would be to use Millipore chamber screening of tumor cells. It should be possible periodically to sample small amounts of blood from your mice, to sift them through Millipore chambers, and to trap the tumor cells that are actually present in a given sample of blood. This could be done at different intervals in both the sea level and the high altitude mice to establish quantitatively whether there are, in fact, more tumor cells per unit of blood per unit time at the high level than at the low level.

Mori-Chavez: I appreciate Dr. Kaplan's suggestions regarding two types of experiments that might help clear up the problem of the differences in metastasis observed between mice at sea level and at high altitude.

Correa:* Dr. Mori-Chavez has shown to us that good research can be done under unfavorable conditions and that it can take advantage of the findings and hypothesis of geographic pathology. The successful growth of metastasis might depend in part on the immune reactions of the host. It is then possible that tumor metastases are more successful at high altitudes because there might be some difficulty with the immune reactions of the animal. Dr. Mori-Chavez, are there any known differences in the responses to antigens at different altitudes?

Mori-Chavez: Not that I know of. This is another interesting and possible factor.

Arias Stella:* Among 152 surgical specimens we have studied from people born and living at Cerro de Pasco (14,300 feet above sea level), we have found 18 cases of cancer. In 185 autopsies performed in the same place we found two cases of carcinoma of the stomach. The malignant neoplasias in the surgical material studied were: epidermoid carcinoma of the cervix (5); stomach carcinoma (2); carcinoma of the gallbladder (1); melanocarcinoma of the skin (3); reticulum cell sarcoma of lymph nodes (2); retinoblastoma (1); mammary carcinoma (1); granulosa cell carcinoma of ovary (1); basal cell carcinoma of skin (1); and 3 examples of metastatic carcinoma of digestive tract origin, primary site not disclosed.

Congdon: It seemed that you emphasized in your report that there was anaplasia observed in the tumor cells, and one suggestion might be that chromosome studies should be done in these peculiar experimental circumstances.

Mori-Chavez: No chromosome studies have been done.

MacCardle:* Anoxic edema occurs at high altitudes. Did this occur in the mice at 15,000 feet? As Zweifach has shown, low oxygen tension removes the intercellular cement (calcium). This may be part of the mechanism of metastasis.

Mori-Chavez: We see edema only in pathological conditions. For instance, in the wound-healing experiment, the edema was much more noticeable at high altitude.

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Cancer Induction by Chemical Substances

Contribution of Urethan Studies to the Understanding of Carcinogenesis^{1, 2}

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SUMMARY

A tremendous increase in our knowledge regarding the carcinogenicity of urethan (ethyl carbamate) has occurred during the past decade. This agent, previously considered a tissue-specific inducer of pulmonary adenoma, is now known to produce neoplasms in a large variety of tissues in several species. Additional experimental findings about a particular carcinogen are welcome, but more important is their potential contribution to the synthesis of generalizations and understanding. In this capacity the investigations with urethan have served well. Urethan has now been added to the list of carcinogens that are multipotential, raising the question as to whether all carcinogens have this characteristic. The examination of this problem requires rigid testing under well-chosen

experimental conditions. Apparently, diverse carcinogens, including urethan, influence the genesis of the same types of neoplasms. Although other factors also play a role, since certain tissues are more prone than others to neoplasms and are sensitive to the action of many carcinogens, the structure, metabolism, and function of a tissue may be determining factors in its becoming neoplastic. Another biologic problem that is worthy of attention relates to whether a carcinogen acts by hastening or precipitating the occurrence of a tumor, *augmentation*, which might normally arise spontaneously in the same site later in the life of the animal, or through *induction de novo*. Evidence and reasoning favoring the former view are presented.—Nat Cancer Inst Monogr 14: 341-356, 1964.

IN THE PAST few decades, there has been an accelerated increase in information regarding the genesis of tumors. On the whole, it has been largely descriptive: studies utilizing new chemical carcinogens in various strains of mice and in other species; data on host and environmental factors capable of modifying carcinogenesis; comparative biochemical and histochemical investigations of normal and neoplastic tissues; morphologic studies with both the light and the electron microscope; and more recently, many studies on radiation and viral carcinogenesis.

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To achieve our goals—the understanding of the nature of carcinogenesis and the control of neoplasms—it is necessary to step up our attempts reasonably to evaluate, interpret, and correlate this extensive literature. It is for this reason that I have chosen to examine briefly the literature on urethan carcinogenesis to extract inferences and concepts which might be integrated with those originating in other areas of investigation. If the objective is achieved, perhaps a small step forward will have been made in our knowledge and understanding of carcinogenesis.

No attempt will be made to present the exact details and results of individual experiments, ours or those of others. Neither will the bibliography be extensive.

INVESTIGATIONS WITH URETHAN

In 1943, Nettleship, Henshaw, and Meyer (1) reported unexpected multiple pulmonary tumors in experimental C3H mice exposed to roentgen rays. They considered and studied the various factors possibly involved and clearly established that the causative agent was the anesthetic, urethan (ethyl carbamate). There followed many investigations, by them and others, which clarified the significance of ethyl carbamate in comparison with other esters of carbamic acid, barbiturates, and other miscellaneous hypnotics [reviewed in (2)]. From both direct studies and consideration of available data, it had been generally accepted that urethan was a tissue-specific carcinogen, one that produced pulmonary adenoma only and no tumors at the site of administration or in other tissues (2, 3).

The resurgence of interest in this relatively simple organic compound (compared with other carcinogens) appears to be based on the demonstration that urethan was not a carcinogen for mouse skin but could so modify it that subsequent application of croton oil resulted in the appearance of papillomas. This was independently shown in 1953 by Graffi, Vlamynck, Hoffmann, and Schulz (4), and Salaman and Roe (5). In 1957, Kirschbaum and Kawamoto (6) demonstrated the potentiating effect of urethan on the induction of mouse leukemia by X rays, and Berenblum and Haran-Ghera (7) considerably augmented, by oral administration of urethan, the small incidence of papillomas of the forestomach found in untreated control mice.

Intrigued by the initial reports concerned with skin carcinogenesis, our laboratory initiated investigations in 1955 on the influence of urethan applied topically to the skin of three strains of mice. In these long-term experiments, we were rewarded by the induction or potentiation of four separate and distinct types of neoplasms: pulmonary adenomas, mammary carcinomas, malignant mesenchymal tumors in the interscapular fat pad, and cystadenomas of the harderian gland. The multipotential carcinogenicity of urethan was so impressive that this fact became the title of the published results (8).

Other reports confirmed the multipotential carcinogenicity of urethan: in the mouse, utilizing other routes of administration (9, 10), in the Syrian golden hamster (11), and in the Sprague-Dawley rat (12). There are many other publications not specifically acknowledged in this brief review. However, more extensive bibliographies are given in the references that are cited.

The neoplasms produced by administration of urethan are shown in table 1. Listed are the main types, in the mouse, rat, and Syrian golden hamster, that have responded to urethan administration by increased incidence, and/or shorter average time of appearance, and increased multiplicity—all indicators of accelerated tumor formation. The list is not complete, and no attempt is being made at this time to evaluate the biological significance of the tumor types that were found in small numbers in the urethan-treated, the control animals, or both. Some representative gross lesions and photomicrographs are shown in figures 1 through 12.

TABLE 1.—Neoplastic response of various tissues to the administration of urethan*

Mouse (various strains)	Rat (Sprague-Dawley)
Pulmonary adenoma	Mammary fibroadenoma
Pulmonary adenomatosis	Sebaceous gland (Zymbal) carcinoma
Pulmonary squamous cell tumor	Malignant lymphoma
Mammary carcinoma	Tumors of kidney
Malignant mesenchymal tumors of interscapular fat pad	Neurilemmoma of ear
Cystadenoma of harderian gland	Hamster (Syrian golden)
Papilloma of forestomach	Melanotic tumors of skin
Hepatoma	Papilloma and carcinoma of forestomach
Leukemia	Adenomatous polyp of cecum
	Pulmonary adenomatosis

*References to publications describing these findings are given in table 3.

The large variety of tissues affected by urethan (and the resulting appearance of neoplasms) suggests that this carcinogen is so versatile that it could be called a polyoma chemical—for the same reason that the polyoma virus was so named. Similarly, irradiation could be considered a polyoma physical agent.

CARCINOGENESIS—INFERENCES AND SPECULATIONS

Having illustrated the increasing knowledge regarding urethan, and the fact that it is a broad-spectrum carcinogen, perhaps we may now turn to the possible impact this information has on the understanding of carcinogenesis. Much is known about chemical and other agents that, under appropriate experimental conditions, influence the formation of tumors. In striking contrast is the paucity of understanding of their mechanisms of action, that is, the intimate, molecular reactions involved in the conversion of normal into neoplastic cells. In our present state of

knowledge, we are not able to detail the stages between exposure to the carcinogenic agent and the onset of neoplasia: the exact site of action in the genetic apparatus, the initial change, and the precise steps; or if one or more mechanisms are involved. It must be recognized, however, that in this respect other pathologic processes (and drug actions) suffer from similar gaps in understanding. It is obvious that we need clearer knowledge not only on a molecular level, but also with regard to the broad, biological aspects. Ideas related to the latter have been expressed previously (8-10, 12) and are extended here.

Are All Carcinogens Multipotent?

It is well known that many carcinogens are multipotent, *i.e.*, capable of producing neoplasms in a variety of tissues. Common examples of these broad-spectrum carcinogens are the carcinogenic polycyclic hydrocarbons, aromatic amines, and irradiation. Recently, urethan has been shown also to be multipotent. The findings with many other agents suggest that they too belong to this category.

May the same be valid for all carcinogens? In all likelihood. However, there has been a tendency mainly to focus attention on carcinogenic action in a particular tissue (as was done with urethan from 1943 to 1953). Experiments are often relatively short term in nature, particularly if the investigator obtains a high yield of the specific neoplasm with which he is concerned.

For the rigid testing of whether or not a particular carcinogen can produce neoplasms in a number of tissues, the experimental conditions have to be chosen with this objective in mind. Consideration must be given to the genetic characteristics of the species and strain; dosage of the carcinogen; route of administration (distribution and concentration of the agent in various tissues); periodicity and duration of treatment; toxicity and effect on metabolism and body weight; age at which treatment is instituted; and the duration of the experiment. We have previously emphasized the importance of excellent laboratory and housing conditions that permit a long, healthy lifespan for animals, so that they may fully express the neoplastic potentialities of the administered carcinogen.

Character of Tissue as a Determinant in Carcinogenesis

It has become apparent that diverse classes of carcinogens may positively influence the genesis of a specific type of neoplasm. Listed in table 2 are a number of carcinogens that induce or potentiate the formation of mammary neoplasms in the rat. Also listed are the many agents that influence the genesis of carcinoma of the sebaceous gland (Zymbal) at the external auditory canal. Supporting evidence for these demonstrations can be found in (12) and in many publications not cited.

These examples, as well as the fact that certain tissues in man and experimental animals are more prone than others to develop neoplasms, sug-

TABLE 2.—Production of rat tumors by diverse carcinogens

Mammary tumors	Sebaceous gland (Zymbal) carcinoma
Estrogen	Benzidine
<i>N</i> -2-Fluorenylacetamide	<i>N</i> -2-Fluorenylacetamide.
4-Aminostilbene	4-Aminostilbene
Irradiation	4-Dimethylaminobiphenyl
3-Methylcholanthrene	3-Methoxy-4-aminoazobenzene
7,12-Dimethylbenz[a]anthracene	7,12-Dimethylbenz[a]anthracene
Urethan (ethyl carbamate)	Urethan (ethyl carbamate)

gest the probability that the structure, metabolism, and function of a tissue may play a determining role in whether or not it becomes neoplastic readily. Clearly, genetic factors are also important, and it is well known that massive exposure to a carcinogen may cause tumors in tissues generally free of these pathologic states. Nevertheless, too little attention has been given to the host factor of tissue susceptibility related to particular structural, metabolic, and functional states.

Carcinogenesis: Induction *De Novo* or Augmentation

Carcinogenesis is the process by which tumors are formed, and a carcinogen is the agent responsible for this action. But how do tumors originate? On the one hand, exposure to an agent may result in the appearance of a neoplasm not found in untreated controls. Here there exists no reason for question or confusion, since the neoplasm has arisen *de novo* and the process may be considered *induction*. On the other hand, when the host utilized is susceptible to a definite frequency, even though low, of spontaneous (normally expected) tumors of a specific type, an agent may produce an *augmentation*, *enhancement*, or *potentiation* of the process, causing a greater frequency of these neoplasms, at a shorter average time of appearance. For clarity and possibly better understanding, these two types of action should be differentiated—qualitatively if possible, but at least quantitatively.

In this connection, a comparison of tumor formation in control and urethan-treated animals is given in table 3. The listing includes 18 neoplasms in three species—the mouse, rat, and Syrian golden hamster. The data are derived from findings in the literature, as well as unpublished data from the author's laboratory. For simplification, the values have been listed or stated in general terms.

With regard to 14 of the 18 neoplasms it is seen that we interpret the action of urethan as that of *augmentation* and not *induction de novo*. This cannot be said about the malignant mesenchymal tumors of the interscapular fat pad of the mouse, the neurilemmoma of the ear of the Sprague-Dawley rat, or the melanotic tumor of the skin and the adenomatous polyp of the cecum of the Syrian golden hamster. The problem as to whether these four neoplasms occur spontaneously requires a more vigorous attack; it is not possible to detail at this time the equivocal factors involved.

TABLE 3.—Comparison of tumor formation in control and urethan-treated animals and evaluation of the mode of action of urethan*

Type of tumor	Nontreated	Urethan-treated	Data in references:	Action of urethan
Mouse (various strains)				
Pulmonary adenoma	Low incidence 2 to 4% 4%	{ Increased incidence Earlier appearance Greater multiplicity 38% 11 to 20% }	Reviewed in (1) (10) (10)	Augmentation
Pulmonary adenomatosis	Low to 100%	{ Increased incidence Earlier appearance Greater multiplicity 5 to 12% }	(8-10)	Augmentation
Pulmonary squamous cell tumor	None	?	(8, 9)	?
Mammary carcinoma	2 to 9% 3 to 7%	{ Increased incidence Greater multiplicity 16 to 43% }	(8, 9, 21) (7)	Augmentation
Malignant mesenchymal tumors of interscapular fat pad	Low to 85%	{ Increased incidence Greater multiplicity Larger size 53 to 80% }	(13, 14, 21)	Augmentation
Cystadenoma of harderian gland	Low to 26%	?	(6, 15-18, 21)	Augmentation
Papilloma of forestomach				
Hepatoma				
Leukemia				
Rat (Sprague-Dawley)				
Mammary fibroadenoma	Approaches 100%	{ Approaches 100% Earlier appearance Greater multiplicity 16% at 62 weeks }	(12)	Augmentation
Sebaceous gland (Zymbal) carcinoma	3% at 115 weeks	16% at 62 weeks	(12)	Augmentation
Malignant lymphoma	1%†	6%	(12)	Augmentation
Tumors of kidney	2% at 112 weeks	4% at 86 weeks	(12)	Augmentation
Neurilemmoma of ear	None	4 to 9%	(12)	?
Hamster (Syrian golden)				
Melanotic tumors of skin	0 to 1%	44%	(11, 19)	?
Papilloma and carcinoma of forestomach	1%	77%	(11, 19)	Augmentation
Adenomatous polyp of cecum	None	11%	(11)	?
Pulmonary adenomatosis	1%	15%	(11)	Augmentation

*Tumor formation, as indicated by incidence and/or average time of appearance and multiplicity of neoplasms.

†Value from two other laboratories.

The question, does a carcinogen hasten or precipitate the occurrence of a tumor that would normally arise spontaneously in the same site later in the life of an animal, is not a new one. It has been raised previously by a number of investigators, but we believe that accumulating evidence gives the concept greater validity. Our own thoughts arose from a consideration of work on the influence of the nutritional state on the genesis of tumors and that concerned with urethan carcinogenesis.

Even if one assumes that all types of neoplasms produced experimentally will be shown to occur spontaneously less frequently, it can be argued that the spontaneous tumors are due to endogenous factors, whereas the additional ones are induced by the carcinogen. One piece of evidence against such an explanation is the data related to pulmonary adenoma in the mouse. Strains having low, moderate, or high incidences spontaneously are influenced in direct proportion, in incidence and multiplicity of tumors, by the administration of urethan. One study with the white-footed field mouse (20) reports a complete absence of pulmonary adenoma in both control and urethan-treated animals. Thus, it appears that the "induction" of such tumors is dependent on a spontaneous tendency for them to arise. These results, therefore, support the augmentation concept, and not one that completely separates the factors that are involved in spontaneous and "induced" tumors.

It seems reasonable and necessary to consider whether preliminary or early neoplastic changes may be taking place in cells, regardless of possible future environmental or experimental exposure to a carcinogen. After all, is not this concept related to those referred to as latent virus, genetic alterations, and modified nucleic acid? All of these are currently receiving, deservedly, much speculative and even some experimental attention.

The distinction between induction and augmentation is not merely an exercise in semantics. Rather, it is an attempt to evaluate the observed phenomena and to clarify our thinking. An increased frequency of tumors—through the application of chemical agents, nutritional factors, hormones, or radiation—poses the problem of interpretation. If we are attempting to penetrate deeper into the nature of carcinogenesis, it is necessary to recognize the way(s) in which an increase in tumor incidence arises and to use terminology that is both descriptive and correct.

RESUMEN

En la década pasada se ha efectuado un avance enorme en nuestro conocimiento sobre la carcinogenicidad del uretano (carbamato de etilo). Este agente, considerado anteriormente como un inductor específico del adenoma pulmonar, se sabe hoy que produce neoplasias en una gran variedad de tejidos de diferentes especies. Todos los hallazgos experimentales que se añadan sobre un carcinógeno particular son bienvenidos, pero más importante es su contribución potencial a la síntesis de las generalizaciones y del conocimiento. En este aspecto las investigaciones con el uretano han sido muy útiles.

El uretano se ha agregado hoy a la lista de los carcinógenos multipotentes, surgiendo el interrogante de si todos los carcinógenos tienen esta característica. Examinar este problema requiere rigurosa prueba bajo condiciones experimentales bien seleccionadas. Se ha hecho evidente que los diversos carcinógenos, incluyendo el uretano, influencian la génesis del mismo tipo de neoplasias. Aunque otros factores también juegan un papel, el hecho de que algunos tejidos son más susceptibles que otros a las neoplasias, y que son más sensibles a la acción de muchos carcinógenos, sugiere que la estructura, el metabolismo y la función de un tejido pueden ser los factores determinantes de su transformación neoplásica.

Otro problema biológico que es digno de atención se refiere a la posibilidad de que un carcinógeno actúa acelerando ó precipitando la ocurrencia de un tumor, *aumento*, que pudiera normalmente ocurrir en forma espontánea en el mismo sitio más tarde en la vida del animal, ó como una inducción *de novo*. Se presentan evidencias y el razonamiento que favorecen la primera opinión.

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PLATE 21

FIGURE 1.—Pulmonary papillary adenoma in a urethan-treated mouse. *Note* vesicular nuclei. Hematoxylin and eosin. $\times 170$

FIGURE 2.—Pulmonary adenomatosis in a control DBA mouse. Hematoxylin and eosin. $\times 300$

FIGURE 3.—Squamous cell pulmonary tumor in a urethan-treated DBA mouse, showing nests of tumor cells in the alveoli. Hematoxylin and eosin. $\times 235$

FIGURE 4.—Nodular squamous cell pulmonary tumor in a urethan-treated DBA mouse, showing large epithelial pearls. Hematoxylin and eosin. $\times 170$

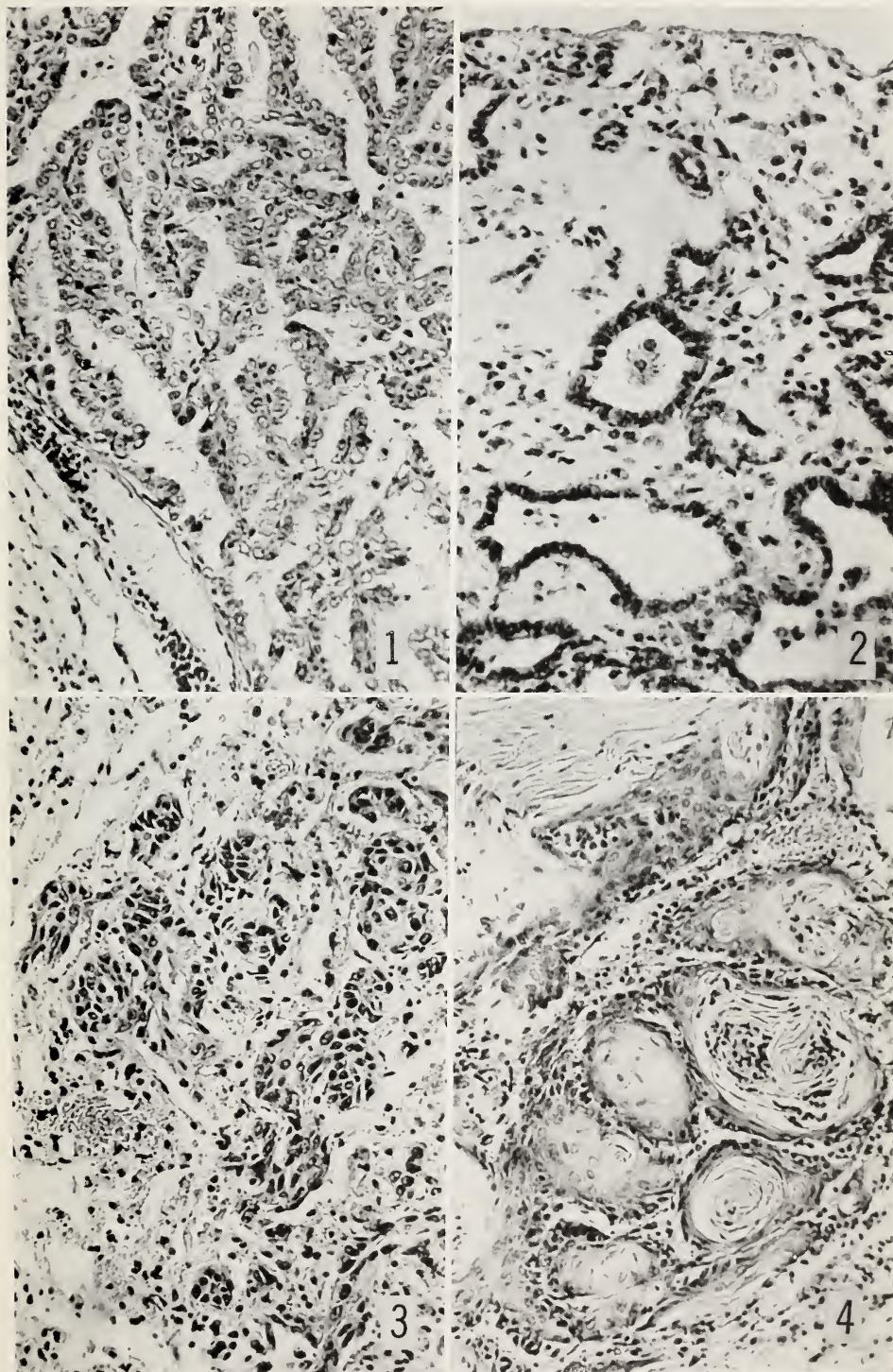


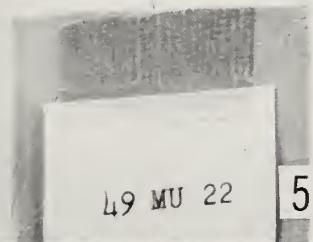
PLATE 22

FIGURE 5.—Urethan-treated $C \times H$ mouse with hemangioendothelioma of the interscapular fat pad.

FIGURE 6.—Hemangioendothelioma of interscapular fat pad in urethan-treated mouse. Angiomatous portion in *upper field*, proliferated endothelial cells in *lower field*. Hematoxylin and eosin. $\times 80$

FIGURE 7.—Bilateral harderian gland adenomas in urethan-treated $C \times H$ mouse. More marked left periorbital swelling.

FIGURE 8.—Papillary cystadenoma of the harderian gland in a urethan-treated $C \times H$ mouse. Normal eye structures in *upper field* and a segment of normal gland on *left*. Hematoxylin and eosin. $\times 55$



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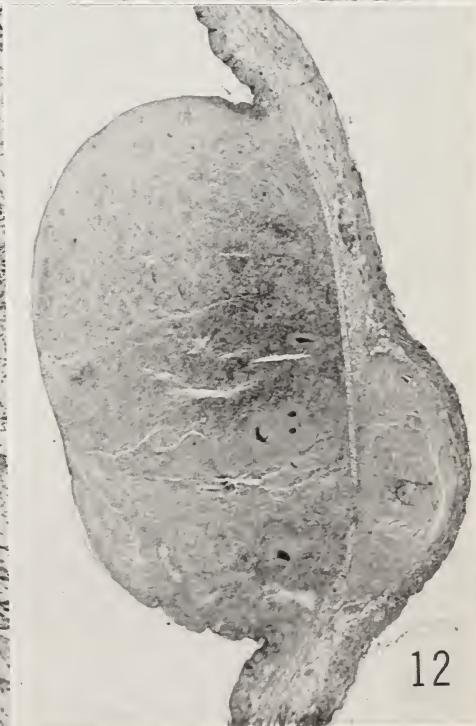
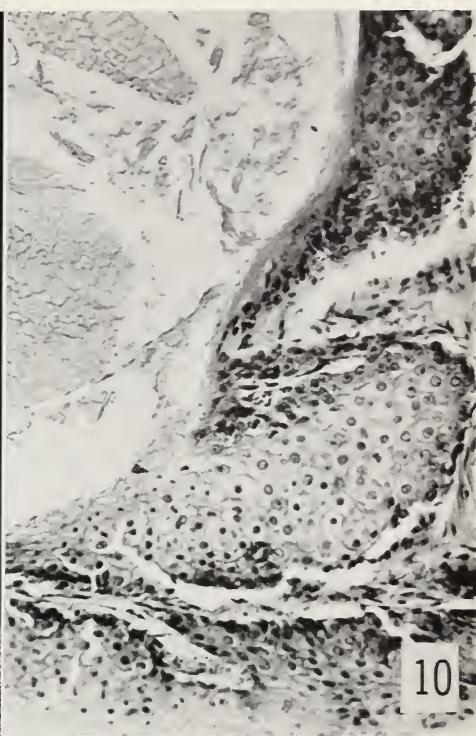
PLATE 23

FIGURE 9.—Tumor of the right sebaceous gland (Zymbal) of the external auditory canal in a urethan-treated rat.

FIGURE 10.—Carcinoma of Zymbal gland in a urethan-treated rat showing central cystic area containing sebaceous material. Hematoxylin and eosin. $\times 170$

FIGURE 11.—Zymbal gland carcinoma in a urethan-treated rat showing squamous cell nests and epithelial pearls. Hematoxylin and eosin. $\times 60$

FIGURE 12.—Neurilemmoma of the ear in a urethan-treated rat. *Note* dumbbell shape of the tumor. Hematoxylin and eosin. $\times 45$



DISCUSSION³

Rogers: If one looks at augmentation in a very broad sense, for example, in relation to the induction of mutation, one arrives at the same conclusion as Dr. Tannenbaum; one cannot say that any specific mutation induced with any of a variety of agents did not even occur spontaneously in the biological population under test, and if the specific mutation does not occur spontaneously in the population under study, then one would not be expected to be able to induce it. This latter possibly bears on the findings that in the white-footed deer mouse (Gross, Cancer 6: 1241, 1953), pulmonary adenomas neither occur spontaneously nor following injection of urethan. Another interesting example is the guinea pig, in which pulmonary adenomas may be readily induced with methylcholanthrene (Heston and Deringer, J Nat Cancer Inst 13: 705, 1952), but not with urethan (Cowen, Brit J Cancer 4: 245, 1950). The latter work apparently relates to the finding that urethan *per se* is not the carcinogen, but that it is converted to or elicits the actual carcinogen (Rogers, J Nat Cancer Inst 15: 1675, 1955). In the rabbit, however, it has not been possible to evoke these tumors with any carcinogen. They are not known to occur spontaneously. If one exposes mouse lung tissue to the action of urethan in the rat, guinea pig, or rabbit, then re-implants it in mice, the tissue exposed in the rat and rabbit develop pulmonary tumors, while that exposed in the guinea pig does not. If, as we have concluded, these pulmonary adenomas are the result of a mutation-like event, then the rabbit would appear to lack the site on its chromosomes for the mutation to occur, the guinea pig the information for the conversion of elicitation of urethan to the carcinogen, and the rat and mouse having both. That certain mutatable sites are particularly sensitive or resistant to specific mutagens is well known from the work of Kølmark (Hereditas 39: 270, 1953) on Neurospora.

Tannenbaum: Dr. Rogers has made some additional interesting comments. In my presentation no attempt was made to discuss even the biological mechanism of carcinogenesis. For example, its complexity is illustrated in situations in which both spontaneous factors and an administered carcinogen influence tumor formation. Under these conditions it may be difficult to determine which has produced the initial change and which the augmentation.

³ Due to technical difficulties in recording, the discussion is incomplete.

Macromolecular Agents as Benign and Malignant Cell Proliferants¹

W. C. HUEPER, *National Cancer Institute,² Bethesda, Maryland*

SUMMARY

The retention of various natural and man-made macromolecular substances (glycogen, lipoprotein complexes, proteins, gum arabic, polyvinyl alcohol, polyvinyl pyrrolidone, methylcellulose, carboxymethylcellulose, pectin, numerous water-insoluble carbon and silicon polymers) in organs and tissues of man and experimental animals has been associated with hyperplastic, blastomatoid and blastomatous manifestations, which often involve cells in which such macromolecular matter is stored. Such correlations are exemplified by hyperglycogenosis with rhabdomyomatosis of the heart, gliomas of the brain, adenomas of the skin, lipoproteinosis with xanthomatosis, lipoidoses with reticulos, proteinoses (Waldenström's hyperglobulinemia, hyperglobulinemia, and Bence-Jones proteinemia) with myelomatosis, reticulum cell sarcoma, lymphosarcoma, and leukemia; asbestos cancers of the lung, pleura, and peri-

toneum, as well as the various macromolecular diseases induced by the parenteral introduction of various natural and man-made, water-soluble and water-insoluble carbon and silicon polymers and plastics with their reticulos, reticulum cell sarcomas, and local sarcomas and carcinomas. They illustrate, moreover, the occurrence of transitions from hyperplastic through blastomatoid to blastomatous reactions. Such observations support the view that the cancers elicited by polymer implants in several species of animals are attributable to the action of chemical factors and do not represent responses to nonspecific surface phenomena. Observations made in macromolecular biology and macromolecular pathology support the view that in some cancers macromolecular chemicals play a significant causal role.—*Nat Cancer Monogr* 14: 357-377, 1964.

AMONG THE diverse, normal and abnormal, endogenous and exogenous agents capable of inducing and maintaining for varying periods benign and malignant cellular proliferations (1), macromolecular chemicals of physiologic and nonphysiologic nature only occasionally have been seriously considered. This relative lack of scientific interest is rather surprising since a considerable amount of evidence, obtained in man and experimental animals, attests to the fact that quantitative and/or qual-

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

tative disturbances of various natural and artifactual macromolecular cellular or humoral constituents are not only associated with local or diffuse proliferations of cells but sometimes are evidently also the primary cause of hyperplastic and neoplastic cellular manifestations (2-5).

ENDOGENOUS HUMAN MACROMOLECULOSES

A number of human macromoleculoses, which in part belong to the general group of thesauroses or storage diseases, illustrates well the biologic principle that normal or abnormal macromolecular compounds of physiopathologic importance may serve as stimulants of benign and malignant cell proliferations.

Glycogen

Glycogenesis, characterized by a congenital inability of the organism to metabolize or depolymerize a physicochemically abnormal glycogen, is associated with accumulation of this polysaccharide in the liver cells and myocardial cells (figs. 1 and 2). These ill-defined nodules in the heart muscle are blastomatoid in character and known as rhabdomyomas. They consist of masses of proliferated, swollen, vacuolated, and distorted muscle cells. It is significant, moreover, that genuine neoplastic manifestations, such as cerebral gliomas and cutaneous adenomas, are often found in individuals affected by this congenital aberration of the glycogen metabolism.

Cholesterol

Hypercholesterolemia, as a hereditary metabolic defect or as the result of endocrine malfunctions (thyroid, pancreas), is associated with the appearance of large lipoprotein complexes in the blood plasma, the development of focal foam-cell proliferations of the arterial endothelium, and of reticuloendothelial cells in various internal organs, such as the liver, spleen, and bone marrow (fig. 3). A frequent additional manifestation of the cellular proliferation stimulating effect of such macromolecular complexes is represented by the multifocal development of xanthomatous tumors mainly affecting the subcutaneous tissue.

Protein

Several proteinotic conditions, such as amyloidosis and the Waldenström's macroglobulinemia, are frequently found in association with neoplastic reactions, such as myelomatosis, reticulum cell sarcoma, and other tumors of the blood-forming tissues. It is unlikely that the abnormal protein metabolites giving rise to the deposition of amyloid are directly involved in the production of neoplastic reactions, since amyloidosis is found also in other diseases associated with an excessive degrada-

tion of proteinic matter, such as tuberculosis of the bones and syphilis. Amyloidosis, moreover, can experimentally be produced in mice by feeding them a diet of cheese or in horses by repeated injections with bacterial vaccines. It is still uncertain, however, whether the specific proteins present in the blood plasma of myelomatosis, and noted in cases of Waldenström's macroglobulinemia, have a direct causal relationship to the various neoplastic diseases frequently coexisting with such abnormal plasma proteinoses. The existence of such interrelations has recently been suggested by observations made on a strain of mice that develops spontaneous myelomas.

TABLE 1.—Endogenous human macromoleculoses

Name	Polymer	Hyperplasias	Neoplasias
Glycogenosis	Glycogen	Myocardial "rhabdomyoma"	Glioma of brain Adenoma of skin
Lipoidosis	Lipoprotein	Endothelial atheromatous cushions Reticuloendothelial Proliferations	Xanthoma
Proteinosis	Amyloidosis Macroglobulinemia (Waldenström)		Myelomatosis Reticulum cell sarcoma Myeloma Leukemia Lymphosarcoma

EXOGENOUS HUMAN MACROMOLECULOSSES

In addition to these observations suggesting a stimulation of benign and malignant cell proliferation under the influence of endogenously produced abnormal macromolecules, there is at least one exogenously elicited macromoleculosis, namely, asbestosis.

Asbestos

Asbestos, which is a polymerized magnesium-iron silicate, induces, when inhaled, the development of a diffuse fibrosing process of the lung, particularly the lower lobes. This condition is complicated in 10 to 50 percent of the cases coming to autopsy with adenocarcinoma or squamous cell carcinoma of the lung (figs. 4 and 5). More recent observations from South Africa, Germany, England, and the United States have, moreover, shown that mesotheliomas of the pleura and peritoneum frequently accompany mild cases of pulmonary asbestosis (fig. 6). The bronchiolar reactions associated with asbestosis consist characteristically of glandular and cystoglandular formations often displaying focal squamous cell metaplasias. It is remarkable that a similar hyperplastic, metaplastic, and neoplastic cellular effect is not exerted by silica.

Beryllium

It is yet uncertain whether the demonstrated formation of protein complexes with various carcinogenic metals, such as chromium, nickel, arsenic, and beryllium, is essential in the production of cancers induced mainly in the organs of the respiratory tract by these minerals. Perhaps the most suggestive evidence existing in this respect relates to beryllium and the pulmonary berylliosis induced by it. Berylliosis is a multifocal sarcoidosis that seems to possess a distinct chemoimmune component in its development. Although there is not as yet any reliable evidence that in man such a condition might lead to cancer, this observation is available for beryllium cancer of the lung experimentally produced in rats and monkeys, and osteogenic sarcoma in rabbits. Beryllium sarcoidosis and cancer thus furnish a suitable subject for investigating the role of macromolecular metal-protein complexes as primary agents in benign and malignant cell proliferation (6, 7).

Polypropylene

The cancers of the nasal sinuses, larynx, and lung observed in producers of isopropyl alcohol, who inhale vapors and mists of the crude liquor (isopropyl oil) from which this alcohol is distilled, may also belong to the group of exogenous polymer cancers. The viscous, somewhat turbid, whitish crude liquor contains polypropylenes, which when exposed to air undergo spontaneous polymerization until they become a dark-brown, tarry mass. It is likely that identical changes occur in the inhaled material when in contact with the air and that the macromolecular polypropylenes thus formed are the carcinogenic agents involved in the causation of the nasal polyps and respiratory cancers seen in such workers.

Natural and Synthetic Gums

During past decades a number of highly polymerized carbon polymers, either of natural derivation, such as gum acacia and pectin, or of semi-synthetic or synthetic origin, such as methylcellulose, carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidone, dextran, iron-dextran, iron-dextrin, and iron-polymaltose, have induced when parenterally introduced into man, animals, or both, not only extensive storage phenomena in various organs such as the liver, spleen, kidney, choroid plexus, bone marrow, and brain, but also extensive and in part blastomatoid and blastomatous proliferations of phagocytic cells in which these macromolecular colloids were retained (figs. 7 through 12). In addition to producing distinct atheromatous foam-cell cushions in the intima of arteries as well as foreign body giant cell granulomas in pulmonary capillaries, these substances elicit a diffuse or focal proliferation of reticuloendothelial cells in the subcutaneous tissue, liver, spleen, and lymph nodes, which in their advanced forms assume blastomatoid proportions. In fact, some of these macromolecular chemicals (polyvinyl pyrrolidone, dextran, polyvinyl alcohol, carboxymethylcellulose, iron-dextran, iron-dextrin, iron-poly-

maltose) ultimately induced cancerous manifestations such as reticulum cell sarcoma, lymphosarcoma, and subcutaneous fibrosarcoma (figs. 13 and 14).

The general types of cellular reactions produced by these extraneous macromolecular colloids resemble morphologically, in many respects, those seen with the various endogenous lipoidoses, such as Niemann-Pick disease, Gaucher's diseases, Tay-Sachs amaurotic idiocy and hypercholesterolemia, and are similar in other respects to the neoplastic manifestations associated with Waldenström's macroglobulinemia. It may be noted that the tumor formation seen after a repeated subcutaneous injection of the three iron-complexes listed cannot justly be ascribed to a carcinogenic action of the iron component (8), but is much more likely due to a specific effect of the macromolecular component of these complexes. Several dextrans have produced cancers in rats, while finely powdered iron, obtained by the degradation of iron carbonyl, when implanted into the pleural cavity of these animals did not elicit a single tumor (9, 10).

The human and experimental evidence presented thus shows unmistakably that a variety of different natural physiologic or pathologic macromolecules, as well as diverse semisynthetic and synthetic water-soluble macromolecular compounds, is capable of stimulating directly or indirectly the proliferation of various types of cells and may sometimes convert normal proliferating cells into malignant ones. Histochemical observations definitely prove that such cellular reactions must be incited by the macromolecular chemicals because these can enter the reacting cells and thus interact directly with intracellular constituents.

TABLE 2.—Exogenous human macromolecules

Asbestosis of lung	Asbestos	Adenomatoid bronchiolar cysts and squamous cell metaplasias	Cancer of lung, mesothelioma of pleura and peritoneum
Local and systemic thesauroses of various water-soluble macromolecular colloids	Gum acacia, pectin, methylcellulose, carboxymethylcellulose, dextran, iron-dextran, iron-dextrin, iron-polymaltose, polyvinyl alcohol, polyvinyl pyrrolidone	Hyperplasia of arterial endothelium, reticuloendothelial system of liver, spleen, lymph nodes, bone marrow, choroid plexus, subcutaneous fibroblasts	Reticulum cell sarcoma, lymphosarcoma, fibrosarcoma (in experimental animals)

EXOGENOUS EXPERIMENTAL MACROMOLECULOSSES

Recent observations on the cell proliferation stimulating and cancer-producing effects of many water-soluble carbon and silicon polymers, commercially known as plastics, suggest that a similar mechanism is apparently operative in the production of these proliferative manifestations. This concept receives special support from the evidence obtained in experiments with implants of several polyurethan foams, because these

macromolecular plastics are readily decomposed in the body of rats by the metabolic forces acting on them (figs. 15 and 16). Both sarcomas and carcinomas develop from the cells surrounding and engulfing the fragments of these polymer foams (11). These observations and those made in connection with the polymer carcinogenesis by other plastics lend little support to the recently advanced hypothesis that these responses are not elicited by chemical factors related to the implanted matter, but rather they represent neoplastic reactions induced by nonspecific factors either associated with some mysterious surface factors emanating from the plastic films or are caused by an interference with the normal metabolism of the cells by large uninterrupted films (12, 13). Under these circumstances, there can be no valid scientific reason for the assumption that sarcomas formed around paraffin pellets implanted in the subcutaneous tissue of mice represent also manifestations of such nonspecific and largely mysterious physical forces (14).

CONCLUSIONS

The human and experimental evidence presented strongly supports the concept that physiologic and pathologic endogenous and exogenous macromolecules can act as cellular proliferants which may induce benign as well as malignant manifestations.

Considering the fact that other macromolecules or gigantomolecules, such as noncarcinogenic and carcinogenic viruses, are well-established cell proliferants and that the carcinogenic effect of ionizing radiations may be related to their depolymerizing or cross-linking action on specific macromolecular cell constituents, the study of animate and inanimate macromolecules indeed represents a fascinating and important facet of cellular physiology and pathology. In fact, the rapidly increasing introduction into the human environment and economy, including medicine, of an ever-growing number and variety of man-made macromolecules and of chemicals capable of influencing polymerization processes in the living tissues present a real challenge to modern biology and public health.

RESUMEN

La retención de diferentes substancias macromoleculares naturales y hechas por el hombre (glicógeno, complejos lipoproteicos, proteínas, goma arábiga, alcohol polivinil, pirrolindon polivinil, metil celulosa, carboximetil celulosa, pectina, numerosos polímeros de carbón y silicón insolubles en agua) en órganos y tejidos del hombre y de los animales de experimentación han sido asociados con manifestaciones hiperplásticas, blastomatoides y blastomatosas que a menudo comprometen las células en las que tales substancias macromoleculares se almacenan. La hiperglicogénesis con rabdomiomas del corazón, los gliomas del cerebro y adenoma de la piel, la lipoproteinosis con xantomatosi, las lipoidosis con reticulocitosis, las proteinosis (hiperglobulinemia de Waldenström, hiperglobulinemia y proteinemia de Bence-Jones) con mielomatosis, sarcoma de células reticulares, linfosarcoma y leucemia; cánceres

por asbestos del pulmón, pleura y peritoneo, así como también las diferentes enfermedades macromoleculares inducidas por la introducción parenteral de diferentes polímeros y plásticos de carbón y silicón solubles e insolubles en agua, naturales y hechos por el hombre, con sus reticulosis, sarcomas de células reticulares y sarcomas y carcinomas locales, son ejemplos de tales correlaciones. Tales ejemplos ilustran, además, las transiciones que ocurren a partir de una reacción hiperplásica a una blastomatoida y una blastomatosa. Tales observaciones apoyan la opinión de que los cánceres provocados por la implantación de polímeros en varias especies de animales son atribuibles a la acción de factores químicos y no representan respuestas a un fenómeno inespecífico de superficie. Las observaciones en la biología y patología macromoleculares apoyan la opinión que en algunos cánceres las substancias químicas macromoleculares juegan un papel causal importante.

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PLATES

PLATE 24

FIGURE 1.—Hepatic glycogenesis showing the vegetable-like liver cells (glycogen stain) (cat).

FIGURE 2.—Rhabdomyoma of the heart with cystic, vacuolated and in part multi-nucleated muscle cells having a foamy cytoplasm (man).

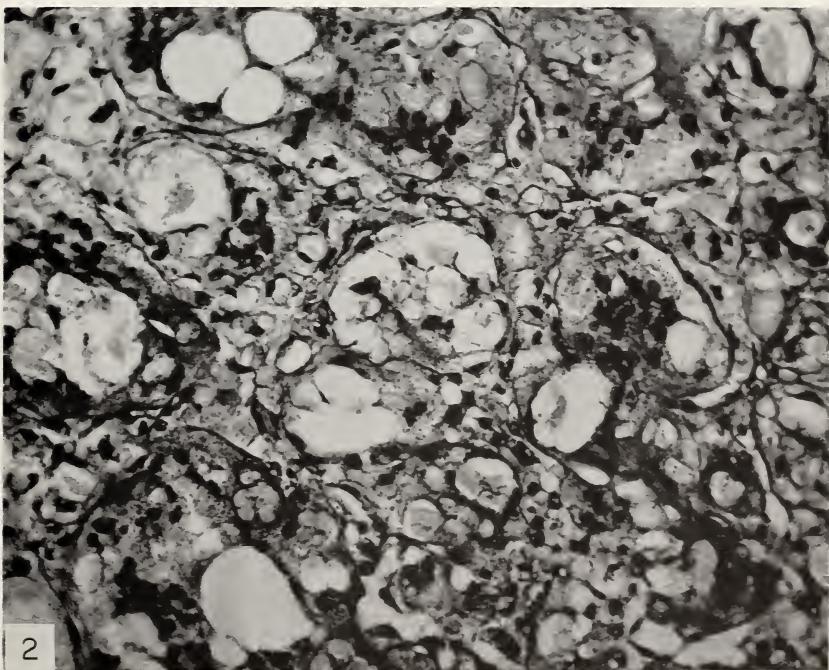
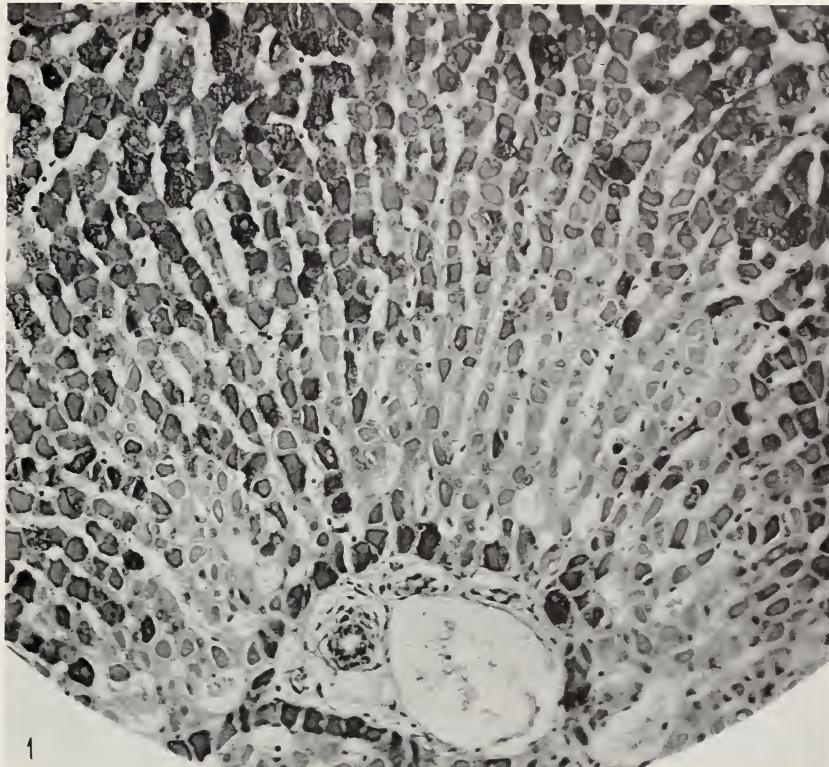


PLATE 25

FIGURE 3.—Intimal foam cell cushion in the aorta of a rabbit fed cholesterol.

FIGURE 4.—Glandular and squamous cell metaplastic lesions in an asbestotic human lung.

FIGURE 5.—Squamous cell carcinoma with asbestos bodies in a human lung.

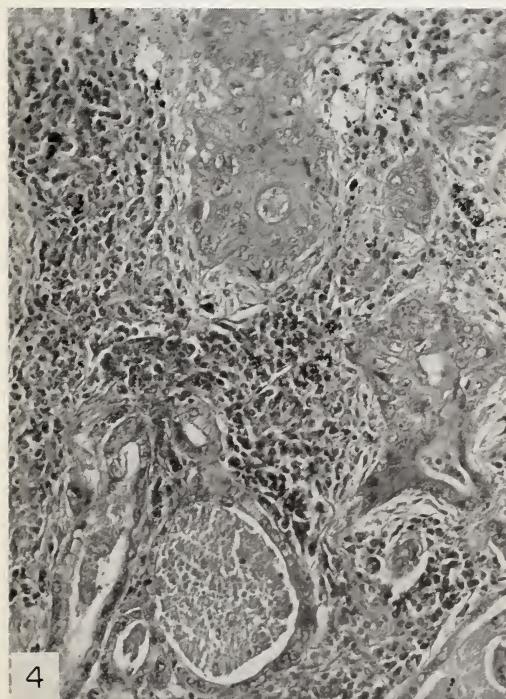
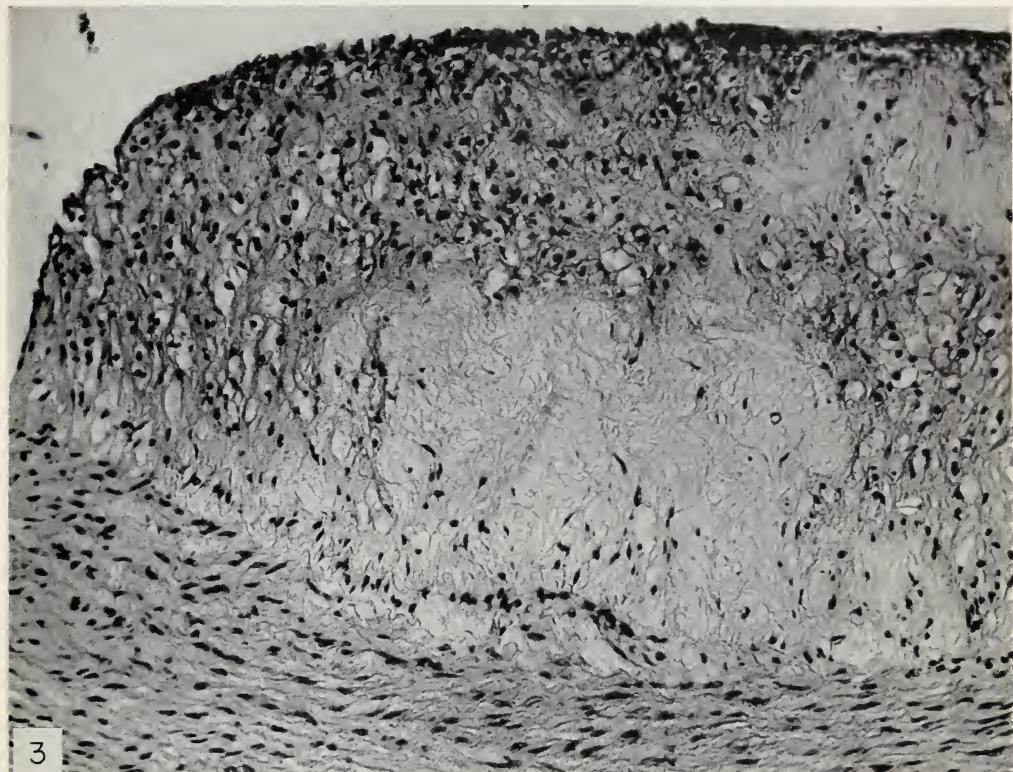


PLATE 26

FIGURE 6.—Mesothelioma of the pleura coexisting with a mild pulmonary asbestosis (man).

FIGURE 7.—Focal endothelial proliferation in the aorta of a dog intravenously injected with methylcellulose solution.

FIGURE 8.—Vacuolated and swollen ganglion and glia cells in the basal ganglion region of a dog intravenously injected with polyvinyl alcohol solution.

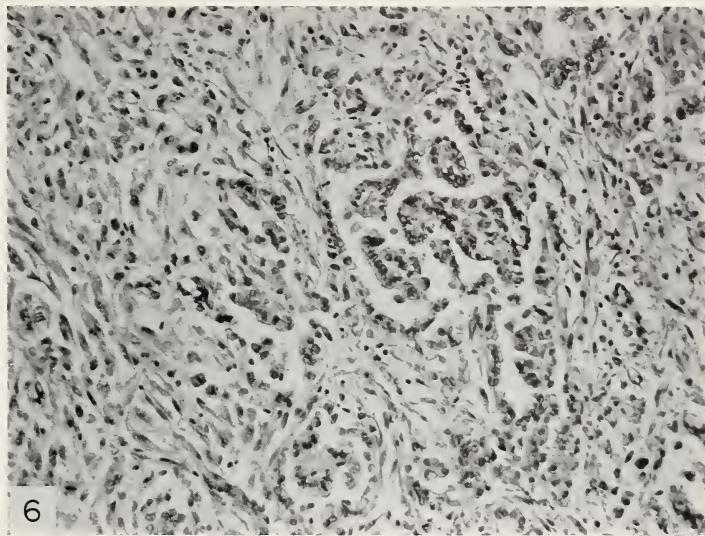


PLATE 27

FIGURE 9.—Proliferated reticulum cells with a foamy cytoplasm in the bone marrow of a dog intravenously injected with methylecellulose solution.

FIGURE 10.—Proliferated choroid plexus cells with a foamy cytoplasm in a rabbit following repeated intravenous injections of a solution of polyvinyl pyrrolidone.

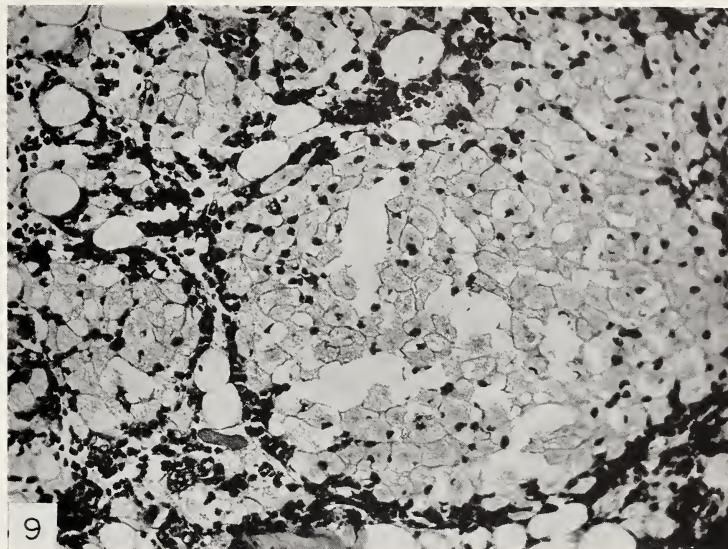


PLATE 28

FIGURE 11.—Blastomatoid proliferation of Kupffer cells in the liver of a dog intravenously injected with methylcellulose solution.

FIGURE 12.—Foam cellular splenic pulp in a dog following repeated intravenous injections of methylcellulose solution.

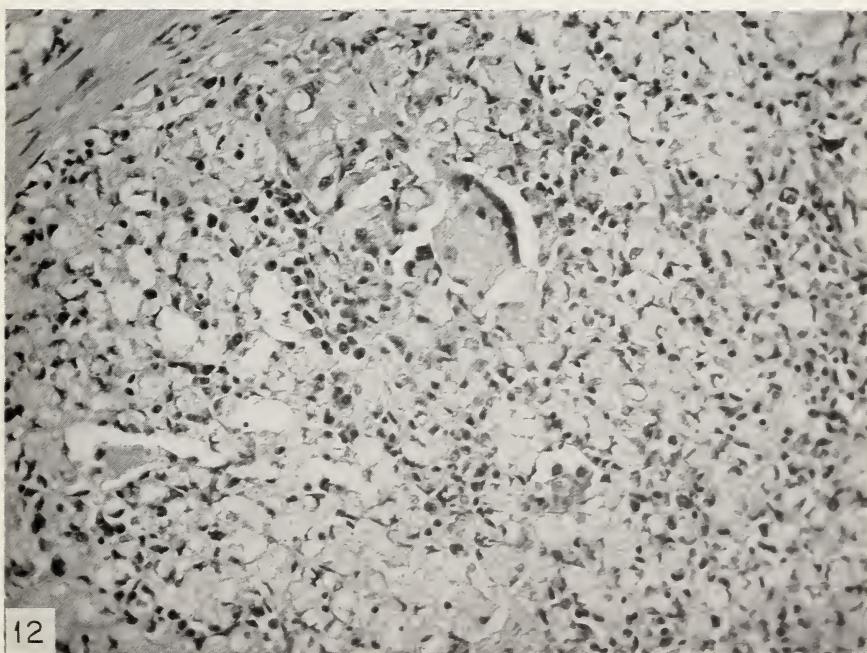
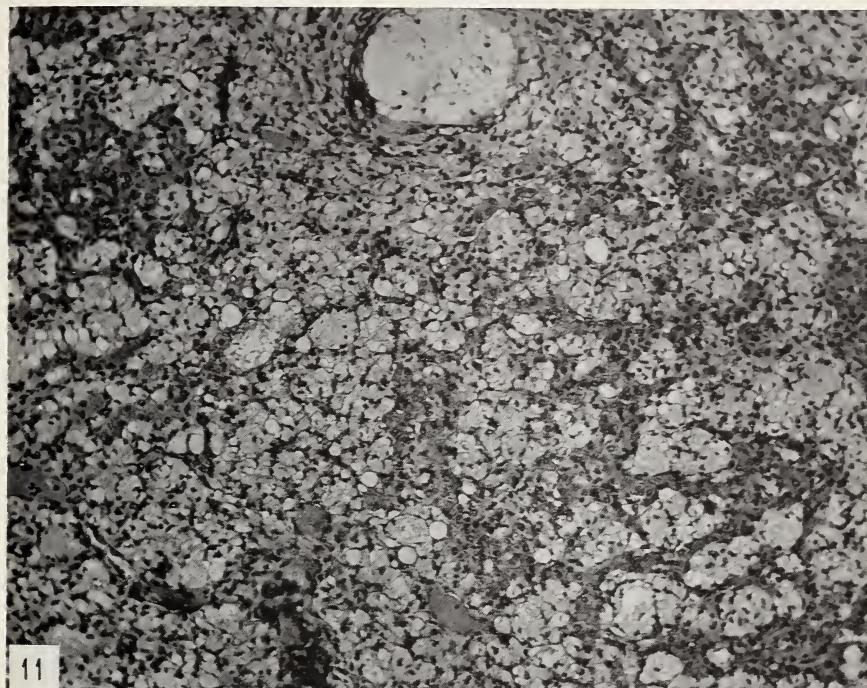


PLATE 29

FIGURE 13.—Kupffer cell sarcoma of the liver of a rat following the parenteral administration of polyvinyl pyrrolidone.

FIGURE 14.—Ileocecal lymph node of a rat with dilated sinuses filled with polyvinyl pyrrolidone surrounded by reticulum cell sarcoma.

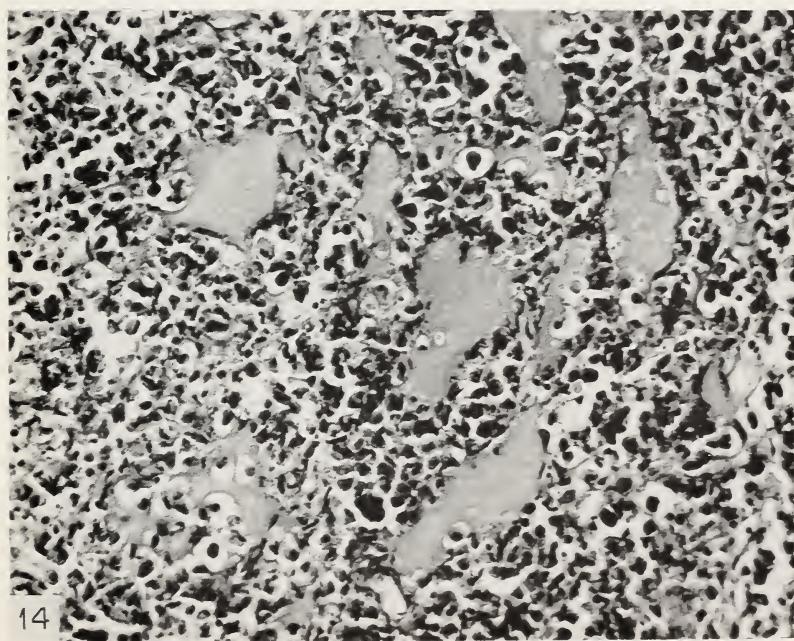
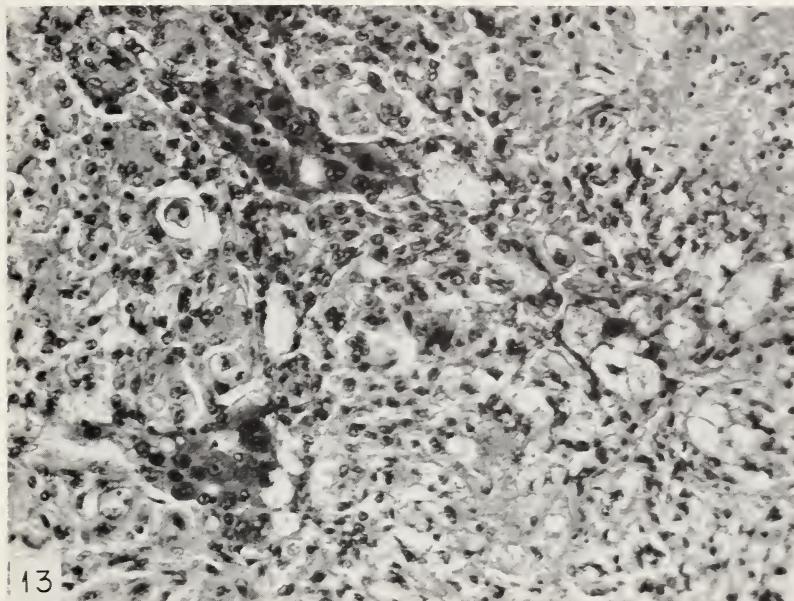
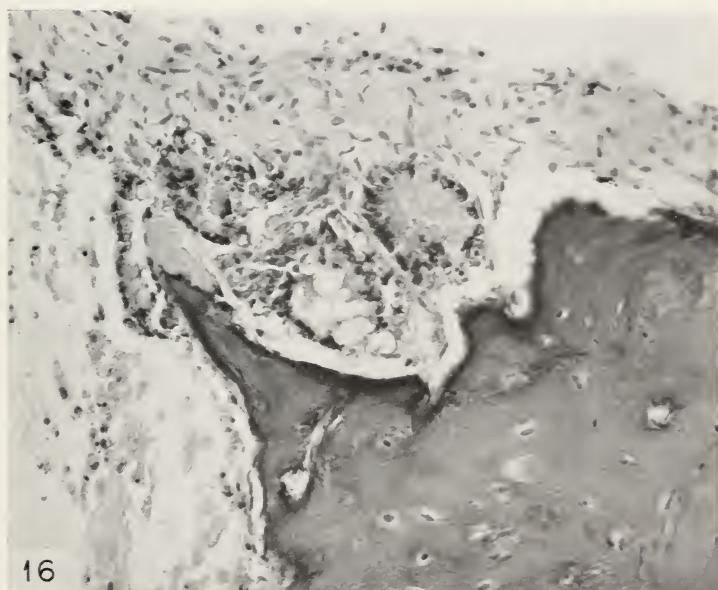
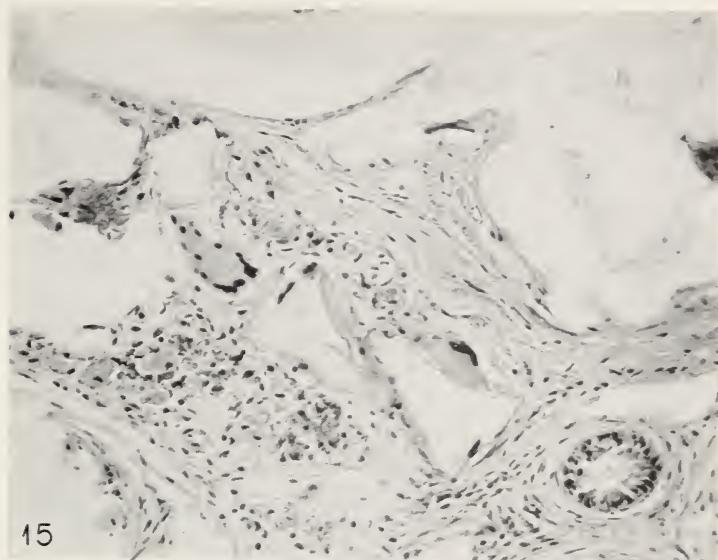


PLATE 30

FIGURE 15.—Pericecal tissue of a rat showing the scaly fragments of an intraperitoneally implanted polyurethan sponge with foreign body giant cells surrounding the scales and large cystic cavities filled with musein in a fibrous stroma.

FIGURE 16.—Gelatinous cylindrical cell adenocarcinoma of the cecum induced by a polyurethan implant with osteoid tissue in the stroma of a rat.



Summary and Final Discussion ¹

CHARLES C CONGDON, *Biology Division, Oak Ridge National Laboratory,² Oak Ridge, Tennessee*

THE THEME of this Conference has been the idea that cancer involves an abnormality in the control of cell division. For this reason the plan of the organizers was to bring two groups of individuals together: those interested in the mechanics and biochemistry of cell division or its control, and those interested in the induction of cancer. We made the assumption that viruses, radiations, chemicals, and all other carcinogenic situations cause cancer by upsetting those mechanisms that control mitosis.

During the 2 years the organizing committees have been arranging the meeting, the idea developed further. It is conceivable that gene control of cell division must be understood to gain insight into the induction of cancer. According to these ideas viruses, radiations, and chemicals interact with those parts of the genome directly concerned with the cell division apparatus itself.

Very little is known about gene control of cell division in any material. Some single factor gene loci are reported, such as the locus for sticky chromosomes in *Zea mays* studied by Beadle (1). Other examples are known, but I think that it is fair to say the basic genetics of the cell division process is unknown to us.

I believe Walter Heston at Bethesda has somewhat similar ideas in mind, as one of several possible mechanisms, in his work on the multiple factor inheritance of cancer with alternative expression of genetic and nongenetic phenomena (2). In our Lima-Cali sessions both D. M. Prescott and Arthur Pardee have probably expressed the Symposium's theme more precisely in referring to the problem as control of nuclear division and the nature of factors that initiate deoxyribonucleic acid (DNA) synthesis. Perhaps it is the gene control of these events that needs our detailed consideration.

Prescott, however, has considered the further point that each gene may have the information necessary for its own replication. In other words, each gene has at least one pleiotropic effect in addition to its usual function.

¹ Presented at the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1-6, 1963.

² Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

It is the opposite point of view to that of one gene or many loci being specifically involved in the control of DNA synthesis.

I would like to turn now to a somewhat different aspect of the Conference theme—to what might be called the three basic principles of the theory of cancer. The best statement of these principles can be found in the writings of the English pathologist Nicholson (3). His collected work, *Studies on Tumour Formation*, is a first-rate summary of many generations of study of the cancer problem by some of the finest scientific intellects in Western Europe and England.

The first principle is that the agents causing cancer are adventitious stimuli. The endless array of chemicals, radiations, viruses, and other phenomena caused cancer adventitiously. There was no rhyme or reason to them. They only initiated something that ended in cancer. H. S. Kaplan referred to this profusion of agents in the introductory remarks to his paper, in Lima.

The second principle in the theory of cancer is that only the responding cells or tissue can develop a cancer. The etiologic agent is not the cancer or any part of it. The old illustration was to point out that painting tar on mice gave rise to malignant skin tumors, but painting tar on the road did not give rise to tumors of the pavement. R. H. Mole, I believe, pointed out during the discussion in Lima that an agent acting on a lung cell would not likely specify the kind of tumor but this would depend on potentialities inherent in the lung cell, not in the agent.

Our present Conference has brought to the forefront the idea that viruses mediate the induction of cancer by all extrinsic or adventitious agents. If we take S. E. Luria's bacteriophage-bacterial cell systems as models for the mechanism of the mediation, then the first two principles—the cornerstones of the basic theory of cancer—disappear. At least, they disappear temporarily. We would probably recover them if we considered stimulus and response situations involving release of latent virus.

The extreme position that all cancer is caused by virus eliminates the adventitious nature of the extrinsic etiologic agent, since the agent now contains specific genetic information in the sense of the bacteriophage systems and that of the Shope papilloma virus discussed by Stanfield Rogers. Further, the second principle does not hold if the virus brings information to the responding host cell genome that changes the nature of the host cell. If this were the case, the lung cell would not necessarily have its inherent potentialities expressed, but might acquire new ones from the virus.

The third basic principle, which Nicholson describes in detail, is that cancer is a biologic phenomenon separated from normal growth and development by an infinite series of gradations. Between cancer and normal growth are all the congenital and acquired tissue malformations. In the cellular tissue changes, from normal growth through malformations to cancer, the idea of cause is also based on multiple nonspecific adventitious stimuli and an inherent cellular response.

If viruses are of major importance in the etiology of all cancer and the third principle is correct, then normal growth and tissue malformations would also be caused by virus-like phenomena. Should the search for viruses in cancer be successful in the extreme sense that I have referred to, then we must look for similar phenomena in normal growth and development and in tissue malformations. The common skin nevus or mole, for example, should have a virus-like etiology.

We might ask Luria and others working on the basic mechanisms of viral action on host cells: What comes before viruses? Are there virus-like phenomena with which we are not yet acquainted? Possibly the findings on "helper viruses" will be the significant direction of research.

I can summarize this portion of my remarks with the comment that if viruses are not acting as adventitious agents then a major change in the classical point of view about the etiology of cancer is necessary.

Another important theoretical development at the Conference was brought out by Pardee and Prescott in their remarks on the evolutionary significance of control of cell division. According to this idea the stopping or controlling of cell division was a process acquired late in evolution to allow differentiation of multicellular organisms. The single-celled organisms divide in an unlimited manner except for limitations set by nutrients and other environmental conditions. This leads us to ask what mechanisms were acquired during evolution to stop the cell division process, or in the view expressed earlier, what genes were acquired to turn off the initiation of DNA synthesis.

In addition to these general remarks on the major themes of the Conference, I would like to call attention systematically to some of the material presented and discussed at the various sessions.

Both Pardee and Herman Kalckar discussed the idea that the point of action or final expression of the carcinogenic agents might be on the surface properties of the cell. Pardee reviewed known surface changes in malignant cells and suggested that control by growth-inhibiting factors from adjacent cells was lacking.

Kalckar extended this concept with the terms "ectobiology" and "ectobiochemistry," defining the latter as the behavior of antigens and receptor sites on cell surfaces. The existence of histocompatibility factors is also a part of this concept. The biochemical entities that Kalckar described were the polysaccharides, which he thought subserved the "social functions" of the cell. He pointed out further that the lack of particular antigens on cell surfaces may be related to an epimerase defect. The Leloir equilibrium is disturbed in tumors with epimerase—a rate-limiting enzyme. Measurements on HeLa and L cells, Ehrlich ascites tumor, and mammary carcinoma support this point of view.

The more frequently considered approach to control of cell division through understanding DNA and ribonucleic acid (RNA) synthesis was presented by Eduardo De Robertis, D. M. Prescott, and Robert P. Perry. De Robertis reviewed the electron microscopy of the nucleus, emphasizing that the basic unit of the chromosome is a filamentous, macromolecular

component or microfibril which may be a single nucleoprotein molecule. Prescott continued the theme, stating that the cell life cycle is a DNA cycle with all events subserving DNA. He believed the question should be asked: What controls initiation of DNA synthesis? This presumably is the point at which carcinogens act or alter DNA in a way that results in cancer. The interrelations of DNA, RNA, and protein synthesis describe the major events of the cell cycle, but Prescott thinks control for DNA synthesis might reside within each gene itself. Chromosomes do not all begin DNA synthesis at once, and the X chromosome is especially known as a late replicator. Perry developed the idea that the nucleolus is a prominent RNA-containing organelle intimately associated with specific chromosomal loci and that its function is a prerequisite for cell division. From Perry's ideas one could develop the thesis that carcinogens upset the usual control of cell division by interfering with normal function of the nucleolus.

A different line of discussion during the Conference was the consideration of specific examples of metabolic control mechanisms in cells. Daniel Mazia mentioned two classes: those that operate by modulating existing cell machinery, and others that create new cell machinery. The first class was essentially equivalent to feedback inhibition of enzyme synthesis illustrated by Pardee. He included in his discussion the two-site model for control of the enzyme, emphasizing in the context of the present Conference that a special site designed for control of the enzyme was present which had nothing to do with the site concerned with the function of the enzyme. Pardee also considered Mazia's second class by illustrating enzyme induction and pointing out in addition the basic concepts of gene function learned from the study of bacteria. The discovery that repressor genes control operator gene function (being able to turn a gene on or off for an extended period) could simulate a mutation in some circumstances, according to Pardee.

Major interest at nearly all cancer meetings centers around the role viruses play in inducing or perpetuating malignant disease. S. E. Luria and Stanfield Rogers dealt exclusively with the actions of viruses on different kinds of systems and, as will be mentioned later, the subject was also considered at length by other participants directly concerned with radiation-induced cancer.

Luria reviewed functional alterations in cells caused by viruses, showing how they may produce permanent changes by initiating new synthetic processes directed by viral genes or by establishing new regulatory controls on the function of cellular genes. Host cell-induced modifications of the virus can also be demonstrated in bacteria. Findings of special interest mentioned by Luria were that viruses can bring a large amount of information to a cell and are capable of mass repression of gene action. They can turn off entire blocks of host genes. Viruses can also specify bacterial cell antigens. The concept of helper viruses that complete the action of a defective virus, such as that seen with the Rous sarcoma, was described by Luria as a potentially significant aspect of tumor viruses.

Rogers presented in detail his work on the Shope papilloma virus. Here the virus brings new antigens to the papilloma and, in addition, new metabolic information in the form of a unique papilloma arginase. Rogers speculated that one approach to the therapy of a genetically determined human disease with loss of an enzyme would be to find a human virus that might transfer the information for producing the enzyme back into the genome.

Since the control of cell division and the induction of cancer must ultimately involve the cells from which the cancer arises, several significant studies on stem cells were presented at the Conference by C. P. Leblond, Joan Wright Goodman, G. S. Hodgson, and L. F. Lamerton. Leblond reviewed the general theory of stem cells and developed the concept of static, expanding, and renewal cell populations encompassing all cells in an individual. Tumors, to a significant extent, resemble expanding and renewal cell populations without the appropriate mechanisms for eliminating the new cells produced. Goodman reported her work on stem cells of the myelopoietic and lymphatic tissues, which, she pointed out, could easily be removed from the animal for study or manipulation *in vitro*, a feature not readily visualized for stem cells of most organ systems. Transplantation studies revealed the presence of hematopoietic and lymphatic tissue stem cells in bone marrow, blood, and peritoneal fluid, whereas lymphatic tissues contained only stem cells for lymphatic tissue replacement, not those of the hematopoietic system.

Hodgson considered the erythropoietic stem cells a specially advantageous model to study control of cell division, since proliferation of these elements can be controlled more or less at will by manipulating endogenous or exogenous erythropoietin. Not only can cell division be initiated by the action of this substance, but it can be stopped by its withdrawal.

Lamerton's work on the ability of stem cells, from various cell renewal systems, to proliferate under continuous irradiation demonstrates the great variability in resistance to injury of mechanisms that control cell division. Presumably, this includes the initiation of DNA synthesis. C. Pavan, however, presented evidence that in some Diptera, where DNA synthesis occurs without cell division, irradiation may actually stimulate DNA synthesis. Others felt Pavan's data reflected a reduction in the pool of endogenous thymidine. Of the systems he studied, Lamerton found the stem cells of the lining of the small intestine most resistant and the testis most sensitive to continuous irradiation. Other cell renewal systems, such as bone marrow, lie between these two extremes.

Radiation-induced cancer was more thoroughly covered at the Conference than any other type of induced cancer. H. S. Kaplan, A. C. Upton, Miriam P. Finkel, and R. H. Mole, respectively, took up the problems of radiation-induced thymic lymphosarcoma, myeloid leukemia, osteogenic sarcoma, and tumors in general, in the mouse. Michael Court Brown discussed radiation-induced leukemia in man.

Kaplan began by pointing out there were two paths the investigator could follow. He could describe the intracellular events essential to the

malignant transformation, or he could follow Kaplan's own approach of defining the pathogenesis by giving the conditions for induction of the neoplasm under study. All five investigators followed the latter course for the most part. Both Upton and Kaplan, however, gave progress reports on their studies with filterable agents probably released by radiation. These agents might conceivably cause leukemia through intracellular events in which a virus is incorporated into the genome of the target stem cell. Upton described very rapid myeloid leukemia induction with cell-free preparations obtained with the Anderson zonal ultracentrifuge, and Kaplan, trying to cope with the variability in assay results that plague work with new leukemia viruses, pointed out the desirability of using a 1-week interval between X-ray exposure and administering the filtrate preparation to the test host.

Finkel suggested in her introductory remarks, before defining the conditions under which radionuclides induce osteogenic sarcoma in mice, that this lesion might turn out to be virus-mediated. Mole was chiefly concerned with complexities in dose-response relations as influenced by the dose rate.

Court Brown described the problem of leukemia in man. A total of 13,000 people with spondylitis irradiated in 32 British radiation centers were studied for induction of leukemia, and many for radiation-induced chromosome abnormalities. In the chronic myeloid leukemic cases Court Brown has investigated, 95 percent showed the Philadelphia chromosome, and a few cases did not. This nearly pathognomonic myeloid leukemia chromosome is not present in other tissues such as fibroblast. Sixty-five percent of the myeloid leukemia cases showed no other chromosome abnormality, but 35 percent had other lesions of the karyotype. Erythrocyte, megakaryocyte, and granulocyte precursors all show the Philadelphia chromosome. There is no difference between the radiation-induced and non-radiation-induced chromosome lesion. Normal cells must be present in a dormant state as they return during remission. During the transition of chronic myeloid into acute leukemia, the Philadelphia chromosome-positive cells increase in numbers. The same chromosomal lesion can presumably be produced by two or more agents. Viruses, such as measles and yellow fever, can cause extensive chromosome breaks *in vivo* and in white blood cell tissue cultures, respectively. Direct preparations of the African Burkitt's lymphoma show chromosome changes. Study of the spondylitis cases revealed stable and unstable abnormalities in the chromosome karyotype. The stable abnormalities persisted for as long as 20 years. One of the questions raised was the significance of the chromosome lesions for the leukemia problem. No clone formation was detected and unstable lines did not persist. Upton pointed out that studies he and Niel Wald were doing on transmitted myeloid leukemia in mice revealed a typical extra chromosome in all the instances examined. This included cases transmitted with cell-free supernatant fluid. Luria remarked in the discussion that

there may be a gene function that keeps chromosomes intact, and this could be unrelated to proliferation.

I also want to mention presentations by H. L. Stewart, Pablo Mori-Chavez, Albert Tannenbaum, and Alexander Hollaender. Stewart quoted the definition of geographic pathology—"Who has what, when, where, and why." He pointed out that less than 1 percent of cancer in man has a known etiology. He gave examples of how geographic pathology dealt with the extrinsic factors in the etiology of scurvy, pellagra, beriberi, and yellow fever and suggested that geographic differences in cancer incidence might similarly provide clues as to the pathogenesis of the tumors in question.

Mori-Chavez found that cancer induction and metastasis of cancer at high altitude differed from those at sea level. The mechanism for these differences was extensively discussed. Arias Stella of Lima and Mori-Chavez both found, contrary to popular belief, that cancer occurred in man at high altitudes in Peru.

Tannenbaum reviewed evidence that demonstrates the multiple carcinogenicity of urethan. This agent produces many different kinds of tumors besides the well-studied pulmonary adenoma. Tannenbaum then asked whether all carcinogens were not multipotential. The data presented showed this to be true. In addition, the same tumor (*e.g.*, mammary tumors in rats) could be produced by several different chemical carcinogens. In Tannenbaum's view, carcinogenesis is an augmentation of spontaneous neoplasia.

Not taken up specifically at this Conference, although mentioned in the discussion, was the possibility that chemical carcinogens release tumor viruses.

Wilhelm Hueper also mentioned some aspects of chemical carcinogenesis in his report on tumor induction by polymers.

Hollaender, in an unusual impromptu session in Lima, described some of the schemes now being considered for stimulating international cooperation in science, including one of establishment of international research institutes to help solve urgent major biological and medical problems, such as the one taken up at this Conference. He encouraged Latin American scientists to participate in these efforts.

CONCLUSION

The Lima-Cali Conference has been an attempt to find significant new ways to think about the induction of cancer, to relate the problem to contemporary basic research in biology, and to re-examine the classical theory of cancer.

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SUMARIO DE LAS ACTAS DEL SIMPOSIO INTERNACIONAL SOBRE EL CONTROL DE LA DIVISION CELULAR Y LA INDUCCION DE CANCER

El tema de esta Conferencia se basa en la idea de que el cáncer implica una anormalidad en el control de la división celular. Por esta razón el plan de los organizadores fué unir dos grupos de investigadores, aquéllos interesados en la mecánica y bioquímica de la división celular o de su control, y aquéllos interesados en la inducción del cáncer. Asumimos que los virus, las radiaciones, los agentes químicos y toda otra situación carcinogénica causan el cáncer perturbando aquéllos mecanismos que controlan la mitosis.

Durante los dos años que los comités organizadores estuvieron preparando la reunión, la idea se desarrolló mejor. Es de concebir que el control genético de la división celular debe ser comprendido para profundizar el mecanismo de la inducción del cáncer. De acuerdo con estas ideas los virus, las radiaciones y los agentes químicos interaccionan con aquellas partes del genoma que están directamente relacionadas con el aparato mismo de la división celular.

Muy poco se sabe sobre el control genético de la división celular en cualquier material. Se han referido localizaciones de genes que gobernan algún factor único tal como el locus para los cromosomas adherentes del *Zea mays*, estudiado por Beadle (1). Se conocen otros ejemplos, pero es justo decir que el fundamento genético del proceso de la división celular nos es desconocido.

Creo que Walter Heston en Bethesda tiene ideas un tanto similares, como uno de los varios posibles mecanismos, en su trabajo sobre los múltiples factores hereditarios del cáncer con expresión alternativa de fenómeno genético y nogenético (2). En nuestras sesiones de Lima y Cali, tanto D. M. Prescott, como Arthur Pardee, han expresado probablemente con más precisión el tema del simposio al referirse al problema como el control de la división nuclear y a la naturaleza de los factores que inician la síntesis del DNA. Es quizás el control genético de estos eventos el que requiere nuestra consideración detallada.

Prescott, sin embargo, ha considerado más allá el punto de que cada gene pueda tener la información necesaria para su propia replicación. Esto quiere decir esencialmente que cada gene tiene por lo menos un efecto pleiotrópico añadido a su función usual. Este es el punto de vista opuesto al de que un gene ó que muchas localizaciones estén implicadas en el control de la síntesis del DNA.

Quisiera tornar ahora a un aspecto un tanto diferente del tema de la conferencia—al que podría denominarse los tres principios básicos de la teoría del cáncer. La mejor presentación de estos principios puede encontrarse en los escritos del patólogo inglés Nicholson (3). Su recopilación de trabajos, *Estudios sobre la Formación Tumoral*, es un sumario de primera clase de muchas generaciones de estudio del problema del cáncer por algunos de los mejores intelectos científicos de Europa Occidental é Inglaterra.

El primer principio es que los agentes causales del cáncer son estímulos fortuitos. El interminable conjunto de agentes químicos, de radiaciones, de virus y de otros fenómenos causaron el cáncer fortuitamente. No hubo rima ó razón para ellos; solamente iniciaron algo que terminó en cáncer. H. S. Kaplan se refirió a esta profusión de agentes en la introducción de su trabajo, en Lima.

El segundo principio en la teoría del cáncer es que solamente las células o tejidos susceptibles pueden desarrollar un cáncer. El agente etiológico no es el cáncer o alguna parte de éste. La vieja comparación señaló que el pintar a los ratones con alquitrán daba origen a cáncer cutáneo, pero que la pista con alquitrán no dió origen a tumores del pavimento. Me parece que R. G. Mole indicó durante la discusión en Lima que un agente que actúa sobre una célula pulmonar no es responsable probablemente de la clase de tumor, sino que éste dependería de las potencialidades inherentes de la célula pulmonar, no del agente.

Nuestra Conferencia ha traído al primer plano la idea de que los virus son los intermediarios in la inducción del cáncer por todos los agentes extríxicos o fortuitos. Si tomamos los sistemas celulares bacteriófago-bacteria de S. E. Luria como modelos para el mecanismo de la mediación entonces los dos primeros principios—piedras angulares de la teoría fundamental del cáncer—desaparecen; por lo menos, desaparecen temporalmente. Probablemente los recuperaríamos si consideramos las situaciones de estímulos y respuestas que impliquen liberación de virus latentes.

La posición extrema de que todo cáncer es causado por virus elimina la naturaleza fortuita del agente etiológico extrínsico, puesto que el agente contiene ahora información genética específica en el sentido de los sistemas del bacteriófago y del virus del papiloma de Shope discutido por Stanfield Rogers. Además, el segundo principio no se mantiene si el virus trae información al genoma de la célula susceptible del huésped que cambia la naturaleza de ésta célula. Si tal fuera el caso, la célula pulmonar no tendría sus potencialidades inherentes expresadas necesariamente, pero podría adquirir nuevas del virus.

El tercer principio básico, que Nicholson describe con gran detalle, es que el cáncer es un fenómeno biológico separado del crecimiento y desarrollo normal por una serie infinita de gradaciones. Entre el cáncer y el crecimiento normal están todas las malformaciones tisulares congénitas y adquiridas. En dichos cambios celulares y tisulares, del crecimiento normal pasando por las malformaciones al cáncer, la idea de causa se

basa también en múltiples estímulos fortuitos noespecíficos y en una respuesta celular inherente.

Si los virus son de mayor importancia en la etiología de todo cáncer y el tercer principio es correcto, luego el crecimiento normal y las malformaciones tisulares serían también causadas por fenómenos viroides. Si la investigación de virus en cáncer fuera exitosa en el sentido extremo que he referido, entonces debemos buscar un fenómeno semejante para el crecimiento y desarrollo normales y para las malformaciones tisulares. El nevo cutáneo común, por ejemplo, tendría una etiología viroide (virus-like).

Una pregunta que debemos hacer a Luria y los otros que trabajan en los mecanismos básicos de la acción viral sobre las células del huésped es “¿que viene antes que los virus?” ¿Existen fenómenos viroides que todavía desconocemos? Posiblemente los hallazgos sobre los “virus cooperadores” (“helper viruses”) serán la dirección importante de la investigación.

Puedo resumir esta parte de mis comentarios anotando que si los virus no actúan como agentes fortuitos entonces es necesario un mayor cambio en el punto de vista clásico sobre la etiología del cáncer.

Otro importante desarrollo teórico de la Conferencia fué ofrecido por Pardee y por Prescott en sus comentarios sobre el significado evolucionario del control de la división celular. De acuerdo a esta idea la detención ó el control de la división celular es un proceso adquirido posteriormente en la evolución para permitir la diferenciación de los organismos multicelulares. Los organismos unicelulares se dividen de un modo ilimitado, excepto por las limitaciones impuestas por los nutrientes y otras condiciones ambientales. Esto nos lleva a preguntar qué mecanismos se han adquirido durante la evolución para detener el proceso de la división celular; ó en la opinión expresada anteriormente, qué genes fueron adquiridos para interrumpir la iniciación de la síntesis del DNA.

Además de estos comentarios generales sobre los temas principales de la Conferencia, quisiera enfocar la atención, en forma sistematizada, sobre algo del material presentado y discutido en las diferentes sesiones.

Tanto Pardee, como Herman Kalckar discutieron la idea de que el punto de acción ó la expresión final de los agentes cancerígenos estaría en las propiedades de la superficie celular. Pardee hizo una revisión de los conocimientos sobre los cambios de superficie en las células malignas, y sugirió que había una falla del control por los factores inhibitorios del crecimiento provenientes de las células contiguas.

Kalckar amplió este concepto con los términos “ectobiología” y “ecto-bioquímica,” definiendo el último como la acción de los antígenos y de los receptores sobre las superficies celulares. La existencia de factores de histocompatibilidad es también una parte de este concepto. Los compuestos bioquímicos que describió Kalckar fueron los polisacáridos que él creía que llenaban las “funciones sociales” de la célula. Remarcó además que la falta de ciertos antígenos en las superficies celulares puede estar en relación con una deficiencia de epimerasa; el equilibrio de Leloir

está perturbado en los tumores con epimerasa, una enzima de acción limitante (rate-limiting enzyme). Mediciones de la escasa actividad de la epimerasa en las células HeLa y las células L, en el tumor ascítico de Ehrlich, y en el carcinoma mamario apoyan este punto de vista.

El enfoque al control de la división celular que se considera con más frecuencia, basado en el conocimiento de la síntesis del DNA y del RNA, fué presentado por Eduardo de Robertis, D. M. Prescott, y Robert P. Perry. De Robertis revisó los estudios del núcleo con el microscopio electrónico, remarcando que la unidad fundamental del cromosoma es un componente macromolecular filamentoso ó microfibrilar, que puede ser una simple molécula de nucleoproteína. Prescott continuó el tema, señalando que el ciclo vital celular es un ciclo del DNA con todos los eventos que conducen al DNA. El se preguntó, "¿qué controla la síntesis del DNA?" Este es probablemente el punto en el cual los cancerígenos actúan o alteran el DNA de un modo que llevan al cáncer. Las interrelaciones del DNA, del RNA, y de la síntesis proteica describen las ocurrencias de mayor importancia del ciclo celular, pero Prescott creé que el control de la síntesis del DNA puede residir dentro del gene mismo. No todos los cromosomas comienzan a la vez la síntesis del DNA, y el cromosoma X es conocido especialmente como un replicador tardío.

Perry desarrolló la idea de que el nucléolo es una organela que contiene RNA asociado íntimamente con las localizaciones cromosomales específicas, y que su función es un requisito previo para la división celular. De las ideas de Perry se podría desarrollar la tesis de que el cancerígeno perturba el control habitual de la división celular, interfiriendo con la función normal del nucléolo.

Una línea diferente de discusión durante la Conferencia fué la consideración de ejemplos específicos de mecanismos de control metabólico en las células. Daniel Mazia mencionó dos clases: aquellos que operan regulando la maquinaria celular existente, y otros que crean nueva maquinaria celular. La primera clase fué esencialmente equivalente a la inhibición de la síntesis enzimática por el mecanismo de regulación retrógrada ilustrado por Pardee. El incluyó en su discusión el modelo de doble localización para el control de la enzima, remarcando en el contexto de la presente conferencia que un lugar designado para el control de la enzima estaba presente, el cual no tenía nada que hacer con el lugar concerniente a la función de la enzima. Pardee consideró también la segunda clase de Mazia ilustrando la inducción de la enzima, señalando además los conceptos básicos de la función de los genes que se conocen por el estudio de las bacterias. El descubrimiento que el represor del control de los genes controla la función del gene operador (pudiendo conectar o desconectar un gene por un período prolongado) podía simular una mutación en algunas circunstancias, de acuerdo con Pardee.

El mayor interés en casi todas las reuniones de cáncer se circunscribe alrededor del papel que juegan los virus en inducir o perpetuar la condición maligna. S. E. Luria y Rogers tratan exclusivamente de la acción de los virus en diferentes clases de sistemas y, como se mencionará más

tarde, el tópico fué considerado también en extenso por otros participantes que se ocuparon directamente del cáncer inducido por radiación.

Luria revisó las alteraciones funcionales de las células causadas por los virus, mostrando como éstos pueden producir cambios permanentes al iniciar nuevos procesos sintéticos dirigidos por genes virales, ó al establecer nuevos controles reguladores sobre la función de los genes celulares. Las modificaciones celulares del huésped inducidas por los virus pueden también demostrarse en las bacterias. Los hallazgos de interés especial mencionados por Luria fueron de que un virus puede llevar una gran cantidad de información a una célula, y que los virus son capaces de una represión masiva de la acción de los genes. Ellos pueden suprimir bloques enteros de genes del huésped. Los virus pueden también dar especificidad a los antígenos bacterianos. El concepto de virus cooperadores que completan la acción de un virus defectuoso, tal como se ha visto con el sarcoma de Rous, fué descrito por Luria como un aspecto potencialmente significante de los virus tumorales.

Rogers presentó en detalle su trabajo sobre el virus del papiloma de Shope. Aquí el virus trae nuevos antígenos al papiloma, y, por añadidura, nueva información metabólica en la forma de una única arginasa del papiloma. Rogers insinuó que una aproximación a la terapia de una enfermedad humana determinada genéticamente con pérdida de una enzima sería encontrar un virus humano que pudiera transferir la información para hacer volver la enzima al genoma.

Desde que el control de la división celular y la inducción del cáncer deben, en última instancia, comprender las células en las cuales se origina el cáncer, varios estudios importantes sobre las células stem se presentaron a la conferencia por C. P. Leblond, Joan Wright Goodman, G. S. Hodgson y L. F. Lamerton. Leblond revisó la teoría general de las células stem y desarrolló el concepto de poblaciones celulares estáticas, expansivas, y renovantes que comprenden todas las células del individuo. Los tumores, en gran parte, se asemejan a las poblaciones celulares expansivas y renovantes sin los mecanismos adecuados para eliminar a las nuevas células producidas. Goodman refirió su trabajo sobre las células stem de los tejidos mielopoyéticos y linfáticos, que precisó que podían ser fácilmente separadas del animal para el estudio ó manipulación *in vitro*, un aspecto no fácilmente visualizado para las células stem de la mayoría de los sistemas orgánicos. Los estudios de transplantes revelaron la presencia de células stem en los tejidos hematopoyéticos y linfáticos en la médula ósea, sangre y fluido peritoneal, mientras que los tejidos linfáticos contenían solamente células stem para el reemplazo del sistema linfático, no del sistema hematopoyético.

Hodgson consideró las células stem eritropoyéticas como un modelo especialmente ventajoso para el estudio del control de la división celular, ya que la proliferación de estos elementos puede ser controlada más o menos a voluntad manipulando la eritropoyetina endógena o exógena. No solamente la división celular puede ser iniciada por la acción de esta substancia, sino que también puede ser detenida retirándola.

El trabajo de Lamerton sobre la habilidad de las células stem de varios sistemas celulares renovantes, de proliferar bajo irradiación continua, demuestra la gran variabilidad en resistencia al daño de los mecanismos que controlan la división celular; probablemente ésto incluye la iniciación de la síntesis del DNA. C. Pavan, sin embargo, presentó datos experimentales de que en algunos dípteros, en los que la síntesis del DNA se realiza sin división celular, la irradiación puede estimular de hecho la síntesis del DNA. Otros creyeron que los datos de Pavan reflejaban una reducción en la cantidad de timidina endógena. En los sistemas estudiados por Lamerton se encontró que las células stem del revestimiento del intestino delgado eran más resistentes y la del testículo más sensibles a la irradiación continua. Otros sistemas de células renovantes, tales como la médula ósea, se sitúan entre estos dos extremos.

El cáncer inducido por radiaciones fué el más cuidadosamente cubierto en la conferencia que ningún otro tipo de cáncer inducido. H. S. Kaplan, A. C. Upton, Miriam P. Finkel y R. H. Mole, se ocuparon, respectivamente, de la inducción por radiaciones de linfosarcoma tímico, de leucemia mieloide, de sarcoma osteogénico, y de tumores en general del ratón. Michael Court Brown discutió la leucemia humana inducida por radiación.

Kaplan comenzó señalando qué habían dos caminos que el investigador podía seguir. El primero, describir los cambios intracelulares esenciales que llevan a la transformación maligna; y el segundo camino, seguido por Kaplan, de definir la patogénesis dando las condiciones para la inducción de las neoplasias en estudio. Todos los cinco investigadores mencionados siguieron el último curso en su mayor parte. Upton y Kaplan, sin embargo, dieron informes de los progresos en sus estudios con agentes filtrables probablemente liberados por radiación. Estos agentes podían probablemente causar leucemia mediante los cambios intracelulares en los cuales un virus es incorporado en el genoma de la célula stem afectada. Upton describió la inducción muy rápida de leucemia mieloide con preparaciones libres de células obtenidas con la ultracentrífuga zonal de Anderson; y Kaplan, tratando de uniformar la variabilidad en los resultados de experiencias que plagan el trabajo con nuevos virus de leucemia, señaló la conveniencia de usar un intervalo de una semana entre la exposición a los Rayos X y la administración de una preparación de filtrado al huésped en prueba.

Finkel sugirió en sus notas de introducción, antes de definir las condiciones bajo las cuales los núcleos radioactivos inducen el sarcoma osteogénico en los ratones, que esta lesión pudiera resultar por la mediación de un virus. Mole se ocupó principalmente de las complejidades en las relaciones de la respuesta a las dosis influenciadas por la tasa de las mismas.

Court Brown describió el problema de la leucemia humana. Un total de 13,000 individuos con espondilitis irradiados en 32 centros británicos de radiación fueron estudiados por inducción de leucemia, y muchos por anormalidades cromosomales inducidas por radiación. En los casos de leucemia mieloide crónica investigados por Court Brown,

95 por ciento mostraron el cromosoma Filadelfia, y muy pocos casos nó. Este cromosoma casi patognomónico de la leucemia mieloide no está presente en otros tejidos tales como los fibroblastos. Sesenta y cinco por ciento de los casos de leucemia mieloide no muestran otras anormalidades cromosomales, pero el 35 por ciento tienen otras lesiones del cariotipo. Todos los precursores de eritrocitos, megacariocitos y granulocitos muestran el cromosoma Filadelfia. No hay diferencia entre la lesión cromosomal inducida y la noinducida por radiación. Las células normales deben estar presentes en un estado quiescente durante la remisión. Durante la transición de leucemia mieloide crónica a leucemia aguda, las células con el cromosoma Filadelfia positivas aumentan en número. La misma lesión cromosomal puede probablemente producirse por dos o más agentes. Los virus, tales como el del sarampión y de la fiebre amarilla, pueden producir cantidad de rupturas cromosomales *in vivo* y en los leucocitos en cultivo de tejidos, respectivamente. Preparaciones directas del linfoma africano de Burkitt muestran cambios cromosomales. Los estudios de casos de espondilitis revelaron anormalidades estables é inestables en el cariotipo cromosomal; las anormalidades persistieron hasta 20 años. Una de las preguntas planteadas fué el significado de las lesiones cromosomales en el problema de la leucemia. No se descubrió la formación de clinos y las líneas inestables no persistieron. Upton, señaló que en los estudios que él y Niel Wald estuvieron realizando en la leucemia mieloide transmitida de los ratones, se encontró un típico cromosoma extra en todos los casos que habían sido examinados hasta entonces; ésto incluyó casos transmitidos con sobrenadantes libre de células. Luria remarcó en la discusión que podría haber una función de los genes que mantiene intactos los cromosomas, y esta no estaría relacionada a la proliferación.

Quiero también mencionar las presentaciones de H. L. Stewart, Pablo Mori-Chávez, Albert Tannenbaum y Alexander Hollaender. Stewart citó la definición de patología geográfica—"Quien tiene qué, cuando, donde y por qué." Señaló que menos del 1 por ciento del cáncer humano tiene una etiología conocida. Dió ejemplos de cómo la patología geográfica trata de los factores extrínsecos de la etiología del escorbuto, de la pelagra, del beri beri y de la fiebre amarilla, y sugirió que las diferencias geográficas en la incidencia de cáncer podían contribuir de un modo semejante a aclarar la patogénesis de los tumores humanos.

Mori-Chávez encontró que la inducción y metastasis de cáncer en las grandes alturas naturales diferían de aquellas al nivel del mar. El mecanismo de estas diferencias fué ampliamente discutido. Tanto Arias Stella, de Lima, como Mori-Chávez observaron, contrariamente a la creencia popular, de que el cáncer humano ocurre en sus diversas formas en las grandes alturas del Perú.

Tannenbaum hizo una revisión de las evidencias que demuestran la múltiple carcinogenicidad del uretano. Este agente produce muchas clases diferentes de tumores, además del bien estudiado adenoma pulmonar. Luego Tannenbaum preguntó si todos los carcinógenos serían multi-

potentes. Los datos presentados indicaron que esto era cierto; más aún, el mismo tumor (por ejemplo, tumor mamario de las ratas) podía ser producido por varios diferentes carcinógenos químicos. En opinión de Tannenbaum, la carcinogénesis es un aumento de la neoplasia espontánea.

La posibilidad de que los carcinógenos químicos liberen los virus tumorales no fué tratada específicamente en esta conferencia, pero sí fué mencionada en la discusión.

Wilhelm Hueper también mencionó algunos aspectos de la carcinogénesis química en su informe sobre la inducción tumoral con polímeros.

Hollaender, en una improvisada sesión, en Lima, describió alguno de los proyectos que se están considerando ahora para estimular la cooperación internacional en ciencia, incluyendo uno sobre el establecimiento de institutos internacionales de investigación para ayudar a resolver grandes problemas biológicos y médicos, tales como los tratados en esta conferencia. Alentó a los científicos Latino Americanos a participar en estos esfuerzos.

CONCLUSION

La Conferencia Lima-Cali ha sido un intento para encontrar nuevas rutas significantes para pensar sobre la inducción del cáncer, para relacionar el problema a la investigación básica contemporánea en biología, y para re-examinar la teoría clásica del cáncer.

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